Vol. 42, No. 4 July, 1955

THE JOURNAL

OF

BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

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THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

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PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published bimonthly, one volume per year.

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The Journal of Biochemistry, Vol. 42, No. 4, 1955

DETERMINATION OF α-KETOGLUTARIC ACID BY COLUMN CHROMATOGRAPHY

By SETSUJI SAKURAI AND KINICHI SUGAE

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(Received for publication, April 11, 1955)

A number of methods for the qualitative and quantitative determinations of keto acids in biological fluids have been published. Shimizu (*1*) described a new method. In this method, the separation of the hydrazones of α-keto acids was carried out by extraction with xylene, which takes only the hydrazones of ketomonocarboxylic acids. Although this method is convenient and gives the satisfactory results in some conditions, it is not advantageous when the sample contains a large quantity of various keto acids. Recently the separation of keto acids by paper chromatography (*2–8*) has been used in this determination. This method, however, is not appropriate in rapid determination. We have found that the determination of α-ketoglutaric acid can be practised by chromatographing its 2,4-dinitrophenylhydrazone on silica gel column. This method is not only convenient, but also very accurate.

In this paper, we describe the method for the determination of α-ketoglutaric acid and some behaviours of two isomers of 2,4-dinitrophenylhydrazone of pyruvic acid.

METHOD

Reagents and Experimental Apparatus

A 5 per cent solution of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid.

Ethyl acetate : Commercial ethyl acetate is shaken with the equal volume of 5 per cent solution of sodium carbonate and then of saturated calcium chloride solution. After being dried over anhydrous potassium carbonate, it is distilled. The distillate collected at 77° was used.

A 10 per cent solution of sodium carbonate.

Column : A glass tube of diameter 10 mm. and length 20 cm.

Silica gel : 4 kg. of water-glass is dissolved in 8 liters of water. To this solution is added concentrated hydrochloric acid with stirring until the pH of the solution has reached 2.0 (this pH value should be maintained for 2 hours at least). The precipitate removed from supernatant and washed with water is suspended in 10 liters of dilute hydrocholric acid (about 1/50 *N*) and then allowed to stand overnight. The precipitate dried at 100° for 48 hours is ground in a moltar and then sifted through 80 mesh sieve.

The Preparation of the Column—A mixture containing butanol, chloroform, acetic acid and water in proportion of 10:90:10:90, 10BC-10A, (this sign is used for abbreviation) are shaken until the two phases are in equilibrium. 1.6 ml. of the upper phase is added to 4 g. of dry silica. After being well stirred with a glass rod, the wet silica is charged into a glass tube and pressed uniformly.

Procedure

About 1 ml. of an aqueous solution (the amount of α-ketoglutaric acid should not excess 1.5 μM. If the removal of protein is necessary, the sample is first deproteinized with 10 per cent sodium tungstate), is added to 1 ml. of solution of 2,4-dinitrophenylhydrazine. After the reaction is allowed to proceed at 37° for 20 minutes, the reaction mixture is poured into a separatory funnel and extracted with 0.7 ml. and 0.5 ml. of ethyl acetate. The combined ethyl acetate solution is placed on the top of a column of silica gel and allowed to penetrate. Then, 10 ml. of the lower phase of the solvent described above is poured slowly on the silica (development usually takes 30 minutes).

After the band of 2,4-dinitrophenylhydrazone of α-ketoglutaric acid is eluted twice with 4 ml of ethyl acetate containing 1 per cent acetic acid, the combined ethyl acetate solution is shaken with 5 mll of sodium carbonate solution in a separatory funnel. After 15 minutes the color intensity of the aqueous layer is measured at about 400 mμ. by photoelectric colorimeter.

Chromatography of 2,4-Dinitrophenylhydrazones of Various Keto Acids

A number of keto acids except α-ketoglutaric, pyruvic, and oxalacetic acid were prepared from the corresponding D-amino acids with the D-amino acid oxidase (*9*, *10*). Fig. 1 gives the chromatograms of 2,4-dinitrophenylhydrazones of various keto acids.

If the sample contains oxalacetic acid, it is difficult to cut the hydrazone of α-ketoglutaric acid exactly. This difficulty, however, can be eliminated when the sample is treated with 0.1 ml. of 1 *M* $CoCl_2$ solution at 37° for 20 minutes prior to incubation with 2,4-dinitrophenylhydrazine.

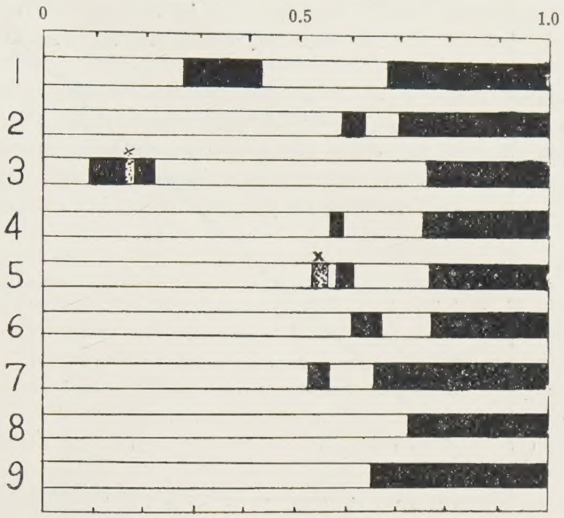
Standard Curve of 2,4-Dinitrophenylhydrazone of α-Ketoglutaric Acid—The standard curve is given in Fig. 2, in which duplicate measurements were carried out.

Recovery Experiments—We obtained a satisfactory result as shown in Table I.

Comparison with the Transaminase Method

Into 20 ml. of the sample (this sample contains oxidative fermentation products of glucose by *Escherichia coli* and α-ketoglutaric acid exists as a calcium salt) was added 1 *N* HCl until the clear solution was obtained and then 1 *N* NaOH was added to bring the pH to 5.0 and the total volume was adjusted to 1500 ml. α-Ketoglutaric acid in 0.5 ml.

FIG. 1. Chromatography of various keto acids.



1. α-Ketoglutaric acid. 2. Pyruvic acid. 3. Oxalacetic acid. 4. Keto acid derived from DL-valine. 5. Keto acid derived from DL-methionine. 6. Keto acid derived from DL-phenylalanine. 7. Keto acid derived from DL-leucine. 8. Keto acid derived from DL-lysine. 9. Calcium-2-ketogluconate.

× Dotted line means a faint band.

TABLE I

Results in Recovery Experiments

Composition / Sample No.	α-keto-glutaric acid	Oxal-acetic acid	Pyru-vic acid	Optical density measured	Concent-ration measured
	μM	μM	μM		μM
1.	0.2	3.0	2.0	0.108	0.23
2.	1.0	3.0	2.0	0.471	1.05
3.	1.5	3.0	2.0	0.670	1.44

of this solution was determined by the method described above and the transaminase method (*5*). As is shown in Table II, there were no significant differences in the results obtained with the two procedures.

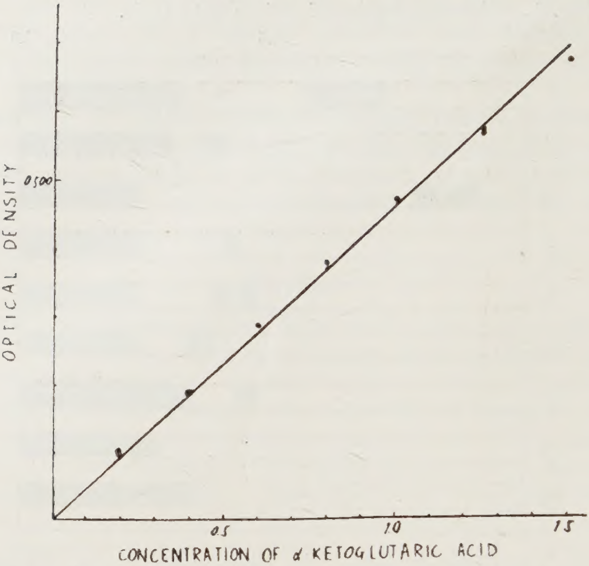


FIG. 2. Standard curve of α-ketoglutaric acid.

TABLE II

Comparison with the Transaminase Method

Method \ Sample No.	The concentration of α-ketoglutaric acid (mg./ml.)		
	1	2	3
Chromatography	0.54	0.50	0.35
Transaminase	0.57	0.49	0.34

On the Two Bands of 2,4-Dinitrophenylhydrazone of Pyruvic Acid

In the course of the study of serine deaminase of *Escherichia coli*, we confirmed that the 2,4-dinitrophenylhydrazone of the deaminated product of DL-serine gave rise to two bands on the column of silica, and that the same bands were obtained when pure pyruvic acid was used. Each of these two bands obtained from 6 μM of pyruvic acid (Rc-value* of the upper bands was 0.19–0.33, the lower was 0.35–0.43 with the solvent, 3BC-3A) was repeatedly treated on the column, until no more two bands appear. After this

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$$* \text{ Rc-value} = \frac{\text{The length from the top of silica gel to the band}}{\text{The length from the top of silica gel to the front}}$$

procedure was repeated five times, each of them was separated completely. The spectral absorptions of these two hydrazones were measured in the 10 per cent sodium carbonate solution by Beckmann's spectrophotometer. (Fig. 3)

Then, in order to obtain these two hydrazones as crystal, we treated 105.6 mg. of sodium pyruvate by the method described above and obtained 18.5 mg. of the upper band (m.p. 204–205°) and 126 mg. of the lower band (m.p. 206–207°) as crystal. These crude crystals were recrystallized by acetic acid and 4.5 mg. from the upper band and 34.5 mg. from the lower band were obtained. The melting point and results of the nitrogene-analysis of these two preparations are given in Table III.

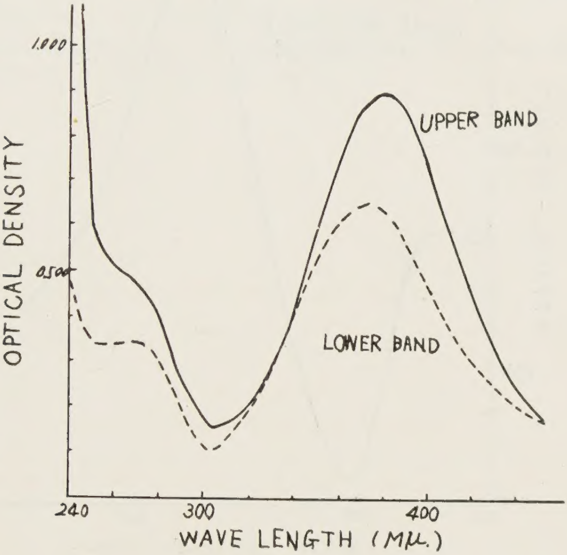

FIG. 3. Absorption curves of hydrozones of pyruvic acid.

The extraction of the both bands were carried out with 5 ml. of 10 per cent sodium carbonate. Ths spectra of the upper band was measured at this concentration, but the lower band was measured after dilution ten times.

TABLE III

Some Behavious of the Preparations Obtained from the Two Bands

Origin	Per cent of nitrogen	Melting point
Upper hand	20.60	213-214°
Lower hand	20.60	213-214°

The spectral absorption of these two preparations was measured at the concentration of 0.17 μ*M* per ml. of 10 per cent sodium carbonate solution. (Fig. 4)

Fig. 4 shows that the absorption curves of these two preparations are the same and have a major absorption at 372 mμ. From these results shown in Fig. 3 and 4, it can be seen that the 2,4-dinitrophenylhydrazone of pyruvic acid usually contains a small amount of unstable form that has a major absorption peak at 380 mμ.

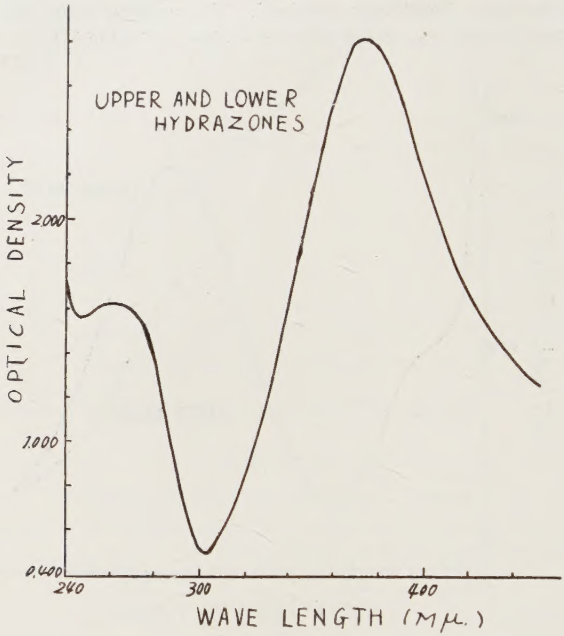

Fig. 4. Absorption curves of two hydrazones of pyruvic acid recrystallized by acetic acid.

SUMMARY

1. A colorimetric determination of α-ketoglutaric acid as 2,4-dinitrophenylhydrazone, separated by silica gel chromatography is described.

2. Some results of experiments on the two isomers of 2,4-dinitrophenylhydrazone of pyruvic acid are described.

3. Comparing this method with the transaminase method, a good agreement was obtained in the determination of α-ketoglutaric acid in oxidative fermentation products of glucose by *Escherichia coli.*

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The Journal of Biochemistry, Vol. 42, No. 4, 1955

STUDIES ON DENITRIFICATION

I. NITROGEN PRODUCTION BY A STRAIN OF DENITRIFYING BACTERIA USING TOLUYLENE BLUE AS A HYDROGEN CARRIER

By HIDEKAZU IWASAKI and TAKESHI MORI

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(Received for publication, April 11, 1955)

As far as is known, the biochemical conversion of nitrate into free nitrogen is a process that is brought about only by some bacteria.

Although a number of researches on this " denitrifying " process have been made (*1–7*), details of its mechanism remain as yet quite obscure.

The present paper describes some experiments upon the denitrifying reaction with special reference to the observation that the process is markedly accerelated by the presence of toluylene blue as a hydrogen carrier.

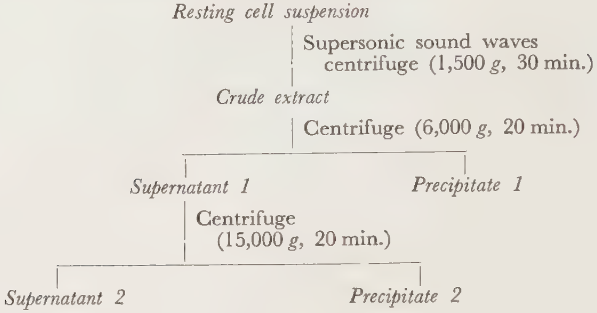
MATERIALS AND METHODS

Microorganism—The bacterium used was originally isolated from soil and has been proved to have a strong activity of producing free nitrogen from nitrate and nitrite. This organism has hydrogenase activity slightly (*cf.* Fig. 3). Analysis of gas produced by the resting cells from nitrite and various carbon sources, e.g. lactate and formate, always showed that nearly 99 per cent of the total volume was N_2 and the rest was methane. The bacteria grown anaerobically for 48 hours at 30° in the peptone-meat extract-0.5 per cent KNO_3 medium containing traces of Mo, Fe, Mn and Cu ions at pH 7 were used.

Resting Cell—The bacteria were harvested, washed two times with saline solution, and suspended in distilled water.

Cell-free Extract—The bacteria cultured under above mentioned conditions were harvested, washed three times with saline solution, heavily suspended in distilled water, and subjected for 20 minutes to supersonic vibration of 500 k.c./sec. which was generated by a 1,000 w. quartz plate oscillator. The cell debris was centrifuged and discarded. The crude extract was then centrifuged at 6,000 *g* for 20 minutes (supernatant 1). Further the supernatant 1 was centrifuged at 15,000 *g* for 20 minutes (supernatant 2). The precipitate was suspended in distilled water of equal volume to that of the supernatant (Fig. 1).

Fig. 1. Fractionation of the bacterial extract.



Preparation of Leuco-toluylene Blue—To 0.1 *M* solution of toluylene blue Pd-$BaSO_4$ was added as a catalyst and after the reduction of the dye by molecular hydrogen, the solution was filtered through a glass filter.

Method of Estimation—Denitrifying activity was measured at 30° in the hydrogen or nitrogen atmosphere in the Warburg apparatus at pH 7 or 7.1.

RESULTS AND DISCUSSION

Using the resting cells, effects of various redox dyes upon the gas evolution were examined as preliminary experiments (Fig. 2).

2,6-Dichlorophenol indophenol, nile blue, gallocyanine and phenosafranine have no effect or inhibit only slightly.

Toluylene blue promotes the rate of gas production remarkably, while the total quantity of gas evolved corresponds fairly to that of nitrite-nitrogen added when toluylene blue is present. Typical experiments carried out in the hydrogen atmosphere are shown in Fig. 3 and similar results were obtained also with nitrogen in the gas phase.

Although the dye was reduced by the action of the dehydrogenase during the equilibration of the reaction system in the manometer vessel, it was oxidized at once, when nitrite was tipped in. These facts indicate indubitably that toluylene blue acted as a hydrogen carrier for the denitrifying reaction.

Then we intended to carry out experiments with cell-free extract of this bacteria using leuco-toluylene blue as the hydrogen donor.

A typical example of the denitrifying reaction with *Supernatant 2* is shown in Fig. 4. Similar results were also obtained with *Supernatant 1.* That is to say, the enzyme system which concerns with the denitrification is contained in the supernatant exclusively.

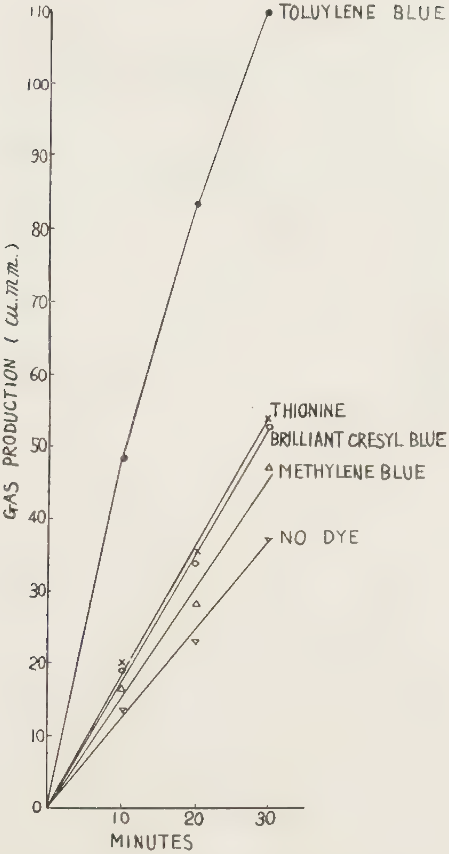


FIG. 2. Effects of various redox dyes.

Activity was measured in the Warburg manometer.

Main cup : resting cell suspension 1 ml. ; sodium lactate $M/2$, 0.3 ml. ; $NaNO_2$, $M/22.4$, 0.3 ml. (13.3 μM) ; phosphate $M/4$, pH. 7, 0.3 ml. Side arm : redox dye 10^{-2} M 0.3 ml. Center well : 20% KOH 0.2 ml. with a piece of filter paper. Total volume : 3.2 ml. Gas phase : hydrogen.

If this system is coupled with some proper dehydrogenase (*e.g.* lactic dehydrogenase), the denitrifying system may be considered to function in the manner as shown in Fig. 5.

Concerning the natural intermediate carrier in the living cell we

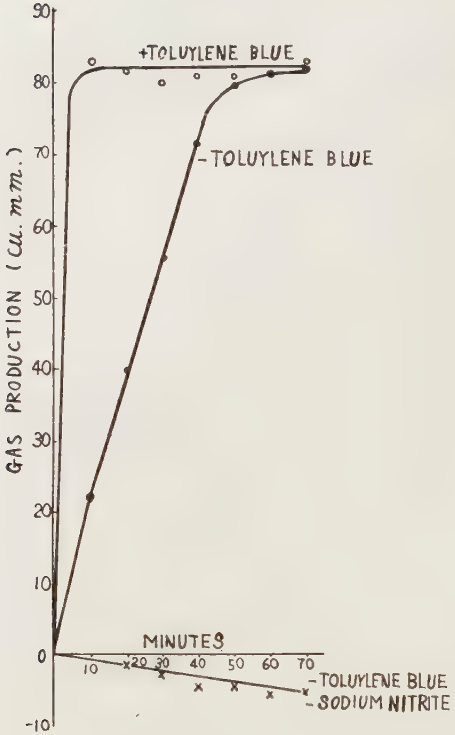


FIG. 3. The quantity of gas evolved from nitrite in the presence of toluylene blue.

Main cup : cell suspension 1 ml. ; sodium lactate $M/2$, 0.3 ml. ; toluylene blue 10^{-2} M, 0.3 ml. ; phosphate $M/4$, pH. 7, 0.3 ml. Side arm : $NaNO_2$ $M/40$, 0.3 ml. (corresponding to 84 cu.mm. N_2/0.3 ml.). Center well : 20% KOH 0.2 ml., with filter paper. Total volume : 3.2 ml. Gas phase : hydrogen.

cannot say anything at present.

The denitrifying activity of *Supernatant 2* is inhibited about 80 per cent by 10^{-3} M KCN and is eliminated by heating at 100° for 10 minutes. Its activity remains unimpaired after dialysis with collodion membrane against distilled water for 2 hours. From the usual experience it may be considered that most part of coenzymes can be eliminated by this process.

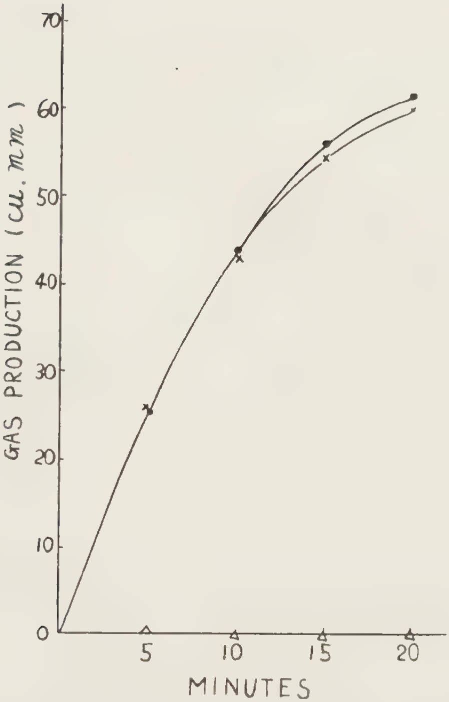
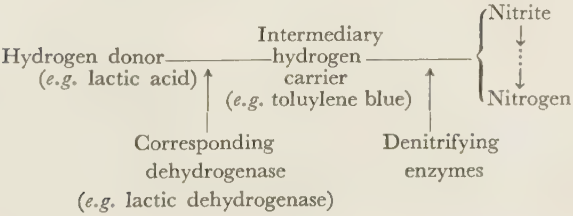


FIG. 4. Gas production from nitrite by *Supernatant 2* and *Precipitate 2.*

Main cup : extract 0.9 ml. ; phosphate *M*/2, pH. 7.1, 0.3 ml. ; leuco-tolyulene blue 0.6 ml. Side arm : $NaNO_2$ *M*/22.4, 0.3 ml. Center well : KOH 0.2 ml., with filter paper. Total volume : 3.2 ml. Gas phase : nitrogen.

● 0.9 ml. *Supernatant 2.* × 0.9 ml. *Supernatant 2*+0.9 ml. *Precipitate 2.* △ 0.9 ml. *Precipitate 2.*

FIG. 5. The mode of action of denitrifying enzymes.



SUMMARY

1. Toluylene blue can act as a hydrogen carrier in the denitrifying reaction.

2. The probable cource of events occurring in bacterial cells was discussed by presenting a tentative scheme of the reaction.

3. The denitrifying enzyme system is contained in the supernatant of cell-free extract obtained by the centrifugation at 15,000 g. for 20 minutes.

4. It is highly probable that some heavy metal participates in the denitrification, because of its pronounced cyanide sensitivity.

5. Denirifying activity is not lost by dialysis.

Thanks are offered to Assist. Prof. Dr. C. Koyama of Chemical Institute, Faculty of Science, Nagoya University for gas analysis.

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ON THE METAPHOSPHATE OF YEAST

III. MOLECULAR STRUCTURE AND MOLECULAR WEIGHT OF YEAST METAPHOSPHATE

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(Received for publication, April 11, 1955)

It has been recognized that metaphosphate of yeast is a condensed phosphate. But we have not yet enough knowledge about the molecular structure and the molecular weight of yeast metaphosphate. About the synthesized inorganic polyphosphates, it has been elucidated that they have chain structures of various polymerisation grades (*1*). On the other hand, Ingelmann et al. extracted from Aspergillus niger a polyphosphate which had a mean molecular weight of 4,000-8,000 (*2*). In yeast cells, there are two kinds of metaphosphates, one of which, " acid soluble metaphosphate ", can be extracted with cold 5 per cent solution of trichloroacetic acid, and the other, " acid insoluble metaphosphate ", cannot be extracted with cold but hot 5 per cent solution of trichloroacetic acid (*3*). These two kinds of yeast metaphosphate seemed to have different biological functions (*3*). It may be considered that their molecular structure are different from each other.

In the present study, two kinds of metaphosphate were extracte: from yeast cells with special attention to avoid the degradation during the extracting process, and their molecular structure and molecular weight were investigated by titration of the weak acid groups and measurement of the viscosity.

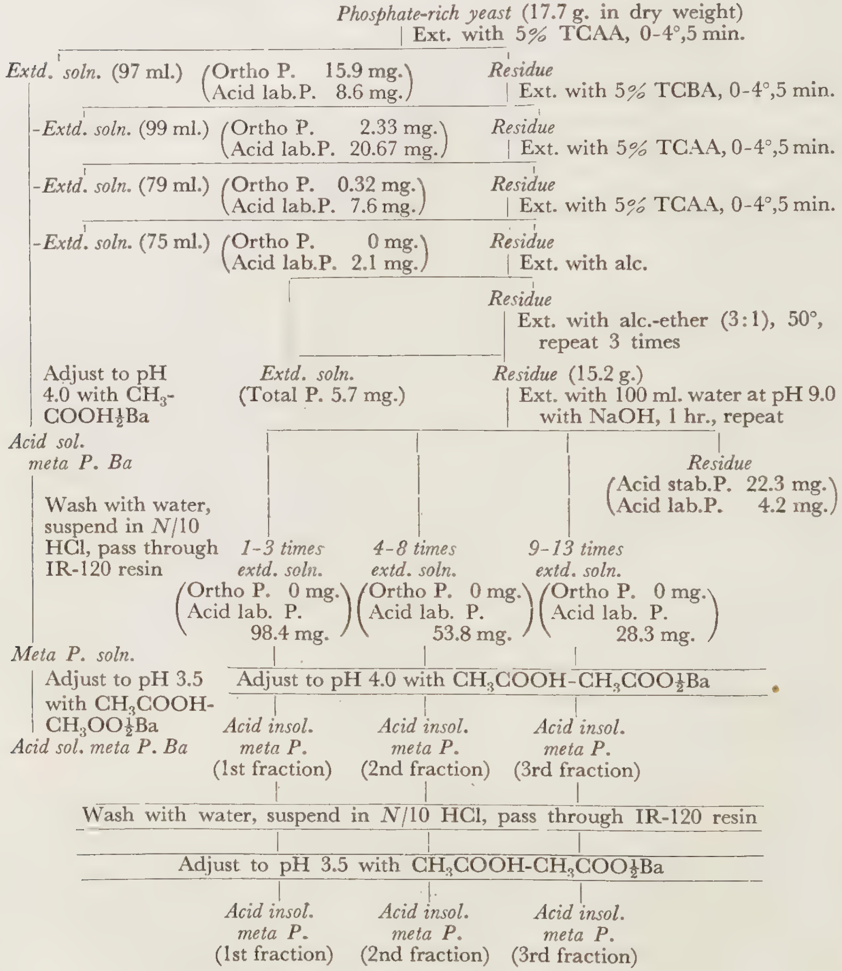
EXPERIMENTAL METHODS AND RESULTS

Extraction and Purification of Yeast Metaphosphate—Phosphate starved yeast cells (*4*) (about 50 g. in wet weight) were suspended in 2000 ml. of media containing 2×10^{-2} M Na_2HPO_4, 2×10^{-3} M $MgSO_4$, 2×10^{-2} M $(NH_4)_2SO_4$, 5×10^{-2} M KCl, 1 per cent solution of glucose and 5×10^{-2} M succinate buffer of pH 5.2, and aerated for 90–120 min. at 30°C. After being treated in this way, the yeast cells were found to contain about 20 mg. of phosphorus per 1 g. of yeast cells (dry weight) ; and they were further washed 4–5 times with 500 ml. of distilled water before the extraction.

Yeast metaphosphate is a acid labile phosphate, and it is necessary to avoid the hydrolysis or degradation of it in the extracting process. It was ascertained that the extracting process did not cause the change of polymerisation grade of synthesized polyphosphate.

The extracting process is shown in a diagram as follows:

Extraction and Purification of Yeast Metaphosphate


Phosphate-rich yeast (17.7 g. in dry weight)
Ext. with 5% TCAA, 0-4°,5 min.
Extd. soln. (97 ml.) (Ortho P. 15.9 mg. Acid lab.P. 8.6 mg.)
Residue
Ext. with 5% TCBA, 0-4°,5 min.
-*Extd. soln.* (99 ml.) (Ortho P. 2.33 mg. Acid lab.P. 20.67 mg.)
Residue
Ext. with 5% TCAA, 0-4°,5 min.
-*Extd. soln.* (79 ml.) (Ortho P. 0.32 mg. Acid lab.P. 7.6 mg.)
Residue
Ext. with 5% TCAA, 0-4°,5 min.
-*Extd. soln.* (75 ml.) (Ortho P. 0 mg. Acid lab.P. 2.1 mg.)
Residue
Ext. with alc.
Residue
Ext. with alc.-ether (3:1), 50°, repeat 3 times
Adjust to pH 4.0 with $CH_3COO\frac{1}{2}Ba$
Extd. soln. (Total P. 5.7 mg.)
Residue (15.2 g.)
Ext. with 100 ml. water at pH 9.0 with NaOH, 1 hr., repeat
Acid sol. meta P. Ba
Residue (Acid stab.P. 22.3 mg. Acid lab.P. 4.2 mg.)
Wash with water, suspend in *N*/10 HCl, pass through IR-120 resin
1-3 times extd. soln. (Ortho P. 0 mg. Acid lab. P. 98.4 mg.)
4-8 times extd. soln. (Ortho P. 0 mg. Acid lab. P. 53.8 mg.)
9-13 times extd. soln. (Ortho P. 0 mg. Acid lab. P. 28.3 mg.)
Meta P. soln.
Adjust to pH 3.5 with CH_3COOH-$CH_3OO\frac{1}{2}Ba$
Adjust to pH 4.0 with CH_3COOH-$CH_3COO\frac{1}{2}Ba$
Acid sol. meta P. Ba
Acid insol. meta P. (1st fraction)
Acid insol. meta P. (2nd fraction)
Acid insol. meta P. (3rd fraction)
Wash with water, suspend in *N*/10 HCl, pass through IR-120 resin
Adjust to pH 3.5 with CH_3COOH-$CH_3COO\frac{1}{2}Ba$
Acid insol. meta P. (1st fraction)
Acid insol. meta P. (2nd fraction)
Acid insol. meta P. (3rd fraction)

(1) Extraction with cold 5 per cent solution of trichloroacetic acid : The phosphate rich yeast cells (50 g. in wet weight) were suspended in cold water, and an equal volume of ice cold 10 per cent solution of trichloroacetic acid was added to it. The extraction was carried out by violent stirring for 5 minutes at 4°. After centrifugation, the pH of the supernatant solution was adjusted to 4.0 by addition of $CH_3COO\frac{1}{2}Ba$. The precipitation of Ba salt of acid soluble metaphosphate was centrifuged after setting in an ice box for one night, and washed three times with water. The residue was extracted again with 100 ml. of ice cold 5 per cent solution of trichloroacetic acid, and the same procedure was repeated three times. After the fourth extraction, orthophosphate and acid soluble metaphosphate were extracted almost completely.

(2) Extraction of lipid : The extracted residue was washed with 100 ml. of alcohol two times using Buchner's funnel. The residue was extracted with 100 ml. of alcohol-ether (3:1) at 50° by using Buchner's funnel and dried in vacuum. With the extracted solutions, total phosphate was estimated.

(3) Extraction with water at pH 9.0 : The lipid extracted residue was suspended in 100 ml. of water, and 2 *N* NaOH was added to it to bring the pH to 9.0 (about 2–3 ml. of NaOH was necessary). After the extraction was carried out by stirring for 1 hour at room temperature, the suspension was centrifuged. The same extracting procedure was repeated until the acid labile phosphate was extracted completely (13–18 times). To the extracted solution, which did not contain orthophosphate, $CH_3COO\frac{1}{2}Ba$ (1 g. per 100 ml. of the extracted solution) and CH_3COOH were added to bring the pH to 4.0. After the mixture was kept in an ice box for one night, the precipitation of Ba salt of acid insoluble metaphosphate was collected by centrifugation and washed three times with water.

The amounts of phosphorus of various fractions are shown in Table I.

Table I

Phosphorus Compounds of Yeast

Phosphate-rich yeast 17.7 g. in dry weight
(after lipid extraction, 15.2 g. in dry weight)

Total phosphate			293.7 mg. P.
Acid sol. P.	ortho P.		18.55 mg. P.
	acid labile P.	pH 4.0 Ba ppt. P.	33.6 mg. P.
		pH 4.0 Ba sol. P.	5.4 mg. P.
Lipid fraction P.			5.7 mg. P.
Acid insol. P.	ortho P.		0 mg. P.
	acid labile P.	pH 4.0 Ba ppt. P.	184.7 mg. P.
		pH 4. 0 Ba sol. P.	0 mg. P.
Alkali insol. P.	acid stable P.		22.3 mg. P.
	acid labile P.		0 mg. P.

(4) Purification of acid soluble and acid insoluble yeast metaphosphate : The

precipitation of B asait of yeast metaphosphate (–100 mg. P) was suspended in ice cold 10^{-1} *N* HCl (about 20 ml.). Ion exchange resine IR-120 (about 4–5 ml.) was added to the suspension and passed through the ion exchange column of IR-120 (about 20 ml.). By this procedure, Ba salt of metaphosphate was changed to metaphospharic acid solution, to which $CH_3COO\frac{1}{2}Ba$ (about 1 g.) and CH_2COOH were added to bring the pH to 3.5. After it had lain in an ice box for one night, the precipitate was centrifuged and washed three times with water. After washing with alcohol and acetone by centrifugation, it was dried in vacuum. The analytical values of P and Ba of acid soluble and acid insoluble yeast metaphosphate were as follows :

Acid soluble metaphosphate	Ba :	P 19.8%	Ba 48.3%
Acid insoluble metaphosphate	Ba :	P 20.3%	Ba 44.7%
Calculated value of $(PO_3\frac{1}{2}Ba)n$		P 20.99%	Ba 46.5%

For the acid group titration metaphosphoric acid solution was prepared by exchanging Ba by H using IR-120 (-H) ; and for the viscosity measurement, sodium metaphosphate solution was obtained by exchanging Ba by Na using IR-120 (-Na).

Molecular Weight of Yeast Metaphosphate by Acid Group Titration—It can be dicided whether yeast metaphosphate would have a chain structure or a ring structure by titration with alkali. If metaphosphate has a chain structure, it should have two weak acid dissociation groups ($pK_a \fallingdotseq 7$), beside strong acid dissociation groups ($pK_a \fallingdotseq 2.0$). On the other hand, acid dissociation groups would be all equivalent ($pK_a \fallingdotseq 2.0$) in polyphosphate having a ring structure.

The yeast metaphosphoric acid solution (15 ml.) was titrated with 10^{-1}*N* KOH against glass electrode. As shown in Fig. 1, there are two kinds of acid groups those dissociation constant are 2.0 and about 7 respectively. From this fact it may be concluded that yeast metaphosphate has a chain structure. Polymerisation grade (n) may be obtained from the titration value by the following equation :

$n = 2 \times P_{eq.}/KOH_{eq.}$

$P_{eq.}$: Phosphorous in metaphosphate solution (in g.)/30.98

$KOH_{eq.}$: Titration value of metaphosphate solution (pH 5.0–9.0)-Titration tion value of dilute HCl 15 ml. (pH 5.0–9.0) (in equivalent)

The polymerisation grades of the various kinds of yeast metaphosphate and "Graham's salt" are shown in Table II. Acid soluble metaphosphate is a chain polyphosphate with a mean polymerisation grade of about 10, and acid insoluble metaphosphate have a mean polymerisation grade of about 50. Both the early extracted acid insoluble metaphosphate and the later extracted one have the same mean polymerisation grade.

Molecular Weight of Yeast Metaphosphate from Viscosity Measurement—The viscosities of synthesized polyphosphates of various polymerisation grades have been measured, and the relationship between polymerisation grade and intrinsic viscosity has been determined (*5*). The viscosity of yeast metaphosphate was measured in 10 per cent solution of tetramethyl-ammonium bromide at 25° using the Ostwald viscosimeter. The polymerisation grade of metaphosphate can be gauged from its intrinsic viscosity by interpolation in Van Wazer's data (*5*). As shown in Table III, the mean poly-

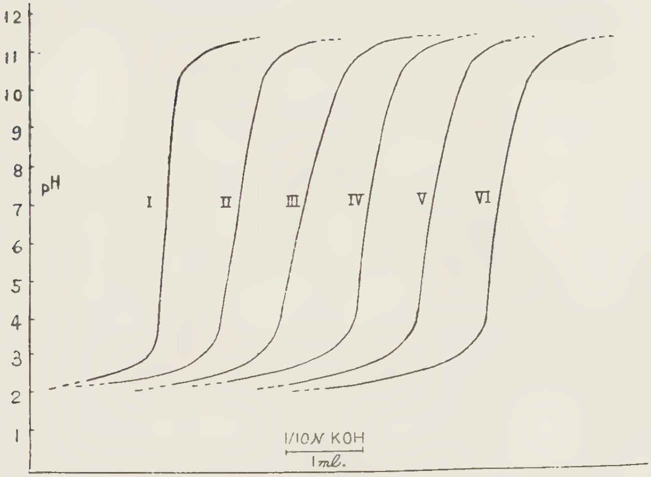


FIG. 1. Titration curve of polyphosphates.

I. dil. HCl. II. Graham's salt P. 6.84 mg. III. Acid sol. Meta. P. 6.61 mg. IV. Acid insol. meta. 1st. fraction P. 13.45 mg. V. Acid insol. meta. 2nd. fraction P. 16.4 mg. VI. Acid insol Meta. 3rd. fraction P. 15.1 mg.

TABLE II

Polymerisation Grade of Yeast Metaphosphate from Weak Acid Group Titration

	Amount of polyphos. in mg. P.	2nd. acid group in $10^{-1}N$ KOH (ml.)	Polymerisation grade
Acid sol. meta P.	6.61	0.400	10
Acid insol. meta P. 1st fraction	13.45	0.175	50
Acid insol. meta P. 2nd fraction	16.4	0.243	44
Acid insol. meta P. 3rd. fraction	15.1	0.170	57
Graham's salt*	6.84	0·220	20

* Graham's salt was the same as that used in determination of heat of hydrolysis in the previous paper.

merisation grade of acid soluble yeast metaphosphate was found to be about 7, and that of acid insoluble yeast metaphosphate about 60. These values agree well with the polymerisation grades determined by titration method.

TABLE III

Polymerisation Grade of Yeast Metaphosphate from Intrinsic Viscosity

	Concentration (C) g./100 ml. soln.	Specific viscosity (η_{sp})	η_{sp}/C	Intrinsic viscosity	Polymerisation grade
Acid sol. meta P.	0.165 0.110 0.055	0.015 0.0103 0.0054	0.090 0.094 0.097	0.10	7
Acid insol. meta P. 1st. fraction	0.171 0.114 0.057	0.034 0.0258 0.0127	0.198 0.226 0.223	0.225	60

Viscosity was measured in 10 per cent solution of tetramethylammonium bromide at 25° using the Ostwald viscosimeter.

DISCUSSION

Polyphosphate may possively have three kinds of structures, a chain structure, a ring structure and or a branched structure. Among these, the branched structure may be considered to be so unstable that can not exist especially in solution (*6*). In this experiment, it has been clarified that the two kinds of yeast metaphosphates are the chain polyphosphates, with polymerisation grades of about 10 and about 50, respectively. The difference of extractability between acid soluble and acid insoluble yeast metaphosphate may be due to the difference of combining strength with proteines. In fact, Katchman *et al.* reported that the higher the polymerisation grade the stronger the combining strength with proteine (*7*).

The greater part of yeast metaphosphate synthesized by intact yeast cells is acid insoluble metaphosphate, and on the other hand, the metaphosphate synthesized from orthophosphate or ATP by the enzyme juice extracted form yeast is all acid soluble one (*8*). The relationship between the structural difference and the physiological function is an interesting problem left for further investigation.

The yeast metaphosphate should be called yeast polyphosphate according to the chemical provision.

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The Journal of Biochemistry, Vol. 42, No. 4, 1955

STUDIES ON CODEHYDROGENASE

I. A SIMPLIFIED METHOD FOR THE PREPARATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE(DPN) FROM BAKERS' YEAST BY CATION EXCHANGE RESINS

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(Received for publication, April 19, 1955)

Many methods for the preparation of diphosphopyridine nucleotide (DPN) have been reported (*1–9*). Most of these methods are complicated by a number of different procedures which are laborious and time-consuming. In 1947, Sumner *et al.* (*7*) introduced a simplified method for the preparation of DPN and this method has been improved by Clark *et al.* (*8*). LePage (*9*) utilized the adsorption by activated carbon for preparing DPN.

Recently, Neilands and Åkeson (*10*), and Kornberg and Pricer (*11*) have employed anion exchange resin in the final stage of purification of DPN. Hogeboom *et al.* (*12*) applied the counter-current distribution technique for the isolation of DPN. This technique is valuable for the preparation of relatively small quantities of highly purified DPN.

The method described here consists of only two main steps — direct adsorption of DPN from a solution extracted from yeast on a special cation exchange resin (*13*) and precipitation of DPN as the silver salt from the eluate. This method is applicable to a large-scale work.

A yield of approximately 1.5 g. of white amorphous material with a purity of about 86 per cent is obtained from 10 kg. of fresh bakers' yeast cake.

EXPERIMENTAL

Determination of DPN—The amount of adenine compounds in the eluate from the resin column was measured by optical density at 260 mμ. Determination of the biological activity of DPN was carried out by the alcohol dehydrogenase methods (*14, 15, 16*) in which the increment of optical density at 340 mμ due to enzymatic reduction was measured.

For the calculation of DPN concentration, the constant of K. Wallenfels and W. Christian (*17*) was used, by whom the extinction coefficient of pure reduced DPN (crystallized as a quinine salt and then decomposed free from quinine with an anion exchanger) at 340 mμ was given as 6.26×10^6 cm.^{2} per mole (9.43 cm.^{2} per mg.).

Extraction of DPN—A vessel containing 8 *l.* of water was heated. When the temperature had reached 95°, curmbled fresh bakers' yeast was added with mechanical stirring as rapidly as possible in such a manner that the temperature was maintained at 80 to 85°. After 10 kg. of yeast had been added, the suspension was kept at about 82° for 10 minutes, and then rapidly cooled to room temperature by a cold water bath.

After the suspension was adjusted to pH 2.0 with concentrated HCl, it was filtered through a large Büchner funnel with suction, employing 200 g. of Hyflo Super-Cel per kg. of yeast. The residue on the funnel was washed with a small volume of water. The slightly cloudy filtrate and the washings were combined.

Preparation of Resin Column—A weak-acid phenol-formaldehyde resin—Kaken cation exchange resin (*13*)—which was prepared by the Scientific Research Institute, Ltd. (Tokyo, Japan) was used. The Na-form resin was ground to 60 to 100 mesh at the swollen state and suspended in about 3 volumes of 2 *N* NaOH at 60° for 2 hours. The supernatant was decanted and the resin was washed several times with a large quantity of water. The washed Na-form resin was twice resuspended in about 3 volumes of 2 *N* HCl. Excess HCl was removed from the resin by washing several times with water. The washed H-form resin was transferred to a glass tube with an inner diameter of 4 cm. and a height of 120 cm.

Adsorption and Elution of DPN—About 15 *l.* of the acidified extract obtained from 10 kg. of pressed yeast was passed through the column of resin mentioned above (4 × 120 cm.) at the flow rate of 500 to 1000 ml. per hour. DPN in the extract was completely adsorbed on the resin. The column was washed with 2 *l.* of 0.02 *N* HCl and successively with 5 *l.* of 0.5 *M* ammonium acetate buffer (pH 4.5) at the flow rate of 250 ml. per hour. These eluates contained no DPN.

The column was mounted on an automatic fraction collector, and DPN was eluted from the resin by passing 0.5 *M* ammonium acetate buffer (pH 9.5 to 10.0) at the flow rate of 250 ml. per hour. The eluate was collected in 60-ml. fractions. Concentrations of total adenine compounds and DPN in each fraction were measured. As will be discussed in detail in the subsequent section, almost all of DPN appeared in the peak of the adenine compounds (see Fig. 2).

Further Purification of DPN by Silver Precipitation—The eluted fractions containing DPN more concentrated than the original yeast extract were combined and neutralized to pH 7.0 with 2 *N* HNO_3. About one-tenth volume of a 25 per cent silver nitrate solution and an equal volume of 95 per cent ethanol were added with stirring, and the resulting precipitate was permitted to settle in a refrigerator. The bulk of the supernatant fluid was decanted and the precipitate was collected by centrifugation. The precipitate was washed twice with 50 ml. portions of a 0.5 per cent silver nitrate solution. The washed precipitate was suspended in about 50 ml. of distilled water and the suspension was acidified with 6 *N* HNO_3 to pH 2.0. Excess hydrogen sulfide was passed into the suspension and the resulting silver sulfide was centrifuged. The precipitate was washed twice with 10 ml. portions of water. The supernatant and washings were combined and aerated to drive off hydrogen sulfide. To the supernatant solution, 2 volumes of 95 per cent ethanol was added with stirring at 5 to 8°, and the precipitate was centrifuged off. To the clear and slightly yellow supernatant, more ethanol was added until the final concentration of alcohol reached 88 volume per cent. The suspension was acidified to pH 2.5 with 6 *N* HNO_3 and chilled overnight at 0 to 5°. The precipitate was collected by centrifugation, washed twice with 95 per cent ethanol, followed with ether, and dried in vacuo over calcium chloride.

RESULTS AND DISCUSSION

Adsorption of DPN on Cation Exchange Resin—As DPN is an ampholyte compound (isoelectric point pH 3.1), it is expected to be adsorbed on both cation and anion exchange resins. In our experiments, DPN was adsorbed on every anion exchange resin tested (Duolite A-4, Duolite A-40, *etc.*) at suitable conditions and on a limited number of cation exchange resins. Various kinds of cation exchange resins including sulfonic polystyrene resins, carboxylic polyacryl resins, and polyphenol resins, were tested. Among them, only polyphenol resins, such as Kaken cation exchange resin, Duolite C-10 and Duolite S-30, showed excellent adsorption capacity for DPN from an acidic solution. From these resins, adsorbed DPN was desorbed with an alkaline solution.

Kaken cation exchange resin is one of porous weak-acid resins which contains two kinds of ion exchange radicals, about 70 per cent of phenoxyacetic acid and 30 per cent of phenol (*13*). Duolite C-10 is an organic sulfonated strong-acid resin, and Duolite S-30 is a super weak-acid resin with phenol as a radical. These resins differ in degree of acidity of ion exchange radicals, but their skeletons are similarly polyphenol.

If the adsorption of DPN on weak-acid polyphenol resins such as Kaken resin occurs by an ion exchange reaction, the adsorbed DPN should be desorbed with a strong acid solution. However, the adsorption occurs more strongly at a lower pH and the desorption of DPN from the resin cannot be effected with concentrated hydrochloric acid. This suggests that the irreversible adsorption of DPN which occurs at a low pH is due to uncharged radicals on the resin, and that the mechanism of desorption at a higher pH is concerned with the conversion of the resin to a salt-form, such as sodium or ammonium. The interaction of DPN and resin may be represented by the following reactions.

$$\text{R-H} + \text{DPN}^+ \longrightarrow \text{R-H} \cdots\cdots \text{DPN}^+ \tag{1}$$

$$\text{R-H} \cdots\cdots \text{DPN}^+ + \text{Na}^+ \rightleftharpoons \text{R-Na} + \text{DPN}^+ + \text{H}^+ \tag{2}$$

It can be supposed that similar reactions occur with other polyphenol resins.

Kaken resin is the most satisfactory for the preparation of DPN compared with other polyphenol resins. Comparing Kaken resin with animal charcoal, the adsorption capacity of DPN on the former is about 5 times larger and the purity of eluate from the former is about 3 times higher than the latter. DPN is easily eluted from the former with an alkaline solution but not eluted from the latter unless treated with pyridine or amyl alcohol.

Relation between Forms of Resin and Adsorption Capacity of DPN—The experiments were carried out on Kaken cation resin. H-, pH 4-, pH 6-, pH 8-, and NH_4-forms of the resin were obtained by treating 0.8-g. portions of H-form resin with 0.5 *M* HCl, 0.5 *M* ammonium acetate buffer of pH 4, pH 6 and pH 8, and 0.5 *M* NH_4OH, respectively. The ion exchange radicals of the buffered resins (pH 4- to 8-forms) contained both H^+ and NH_4^+ in proper proportions.

Each resin was transferred to a column with an inner diameter of 0.5 cm. and then, yeast extracts adjusted to pH 3, 4, 6, 8, and 11 with NH_4OH or HCl were passed through the corresponding pH-columns at the flow rate of 15 ml. per hour. The amounts of DPN in the solutions from each column are given in Fig. 1.

As shown in Fig. 1, both pH 8- and NH_4-forms of the resin scarcely adsorbed DPN, while H- and pH 4-forms strongly adsorbed it from the solutions of each pH.

Elution of DPN—Though DPN is desorbed from cation exchange resins on conversion of the resin to sodium or ammonium form by flow of a solution of NaOH or NH_4OH, DPN is eluted together with many impurities such as flavin compounds and nucleotides other than DPN by these procedures. As shown in Fig. 2, when the column adsorbing DPN is washed with 0.5 *M* ammounium acetate buffer (pH 4.5), considerable amounts of adenine compounds and colored matter are eluted, but DPN is not eluted. Almost all of DPN is eluted with 0.5 *M* ammonium acetate buffer (pH 9.5 to 10.0), and bulk of flavin compound remains in the resin. The fractions desorbed with 0.5 *M* NH_4OH after elution with the buffer of pH 9.5, contain little DPN. These elution processes with various buffers are shown in Fig. 2 in one-

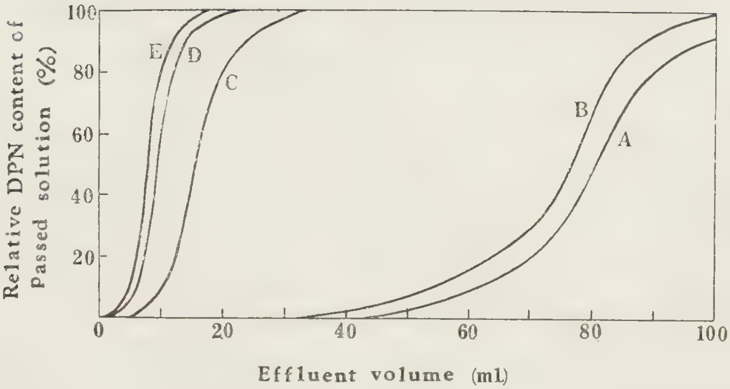

FIG. 1. Adsorption of DPN on different forms of Kaken cation exchange resin.

Dry weight 0.8 g. portions of the resin (column of 0.5 × 3.5 cm.) were used. Yeast extracts adjusted to each pH of the resin were passed through at the flow rate of 15 ml. per hour. Curves show the amount of DPN passed through the column of resin of the following forms : A, H- : B, pH 4- : C, pH 6- : D, pH 8- : E, NH_4-.

fourth scale of our standard method, and the ratios $E^{red}_{340m\mu}/E^{oxi}_{260m\mu}$ for the signed fractions of the main peak in Fig. 2 are shown in Table I.

The fractions containing DPN, fraction number 36 to 61 in Fig. 2, were combined, and further purifications by silver nitrate and ethanol precipitation were performed. The final product was a white amorphous material, and a yield of approximately 0.35 g. of material with a purity of 86 per cent was obtained from 2.5 kg. of fresh yeast.

Modified Method—The standard method described above is of advantage for the preparation of highly purified DPN. For the rapid preparation of partially purified DPN in a high yeild, the following modified method is profitable. DPN adsorbed on Kaken cation exchange resin as above is eluted with 1 *M* NH_4OH without any washing. Thus, DPN in the extract from 10 kg. of fresh yeast is concentrated to about 500 ml. From this eluate, 3 to 5 g. of DPN with a purity of 30 to 40 per cent is obtained merely by fractionation with ethanol, and 2 g. of DPN with a purity of 60 to 70 per cent by the silver treatment and ethanol fractionation.

It is possible to obtain a purer product (about 90 per cent) by the standard method by collecting the fractions in which DPN is contained

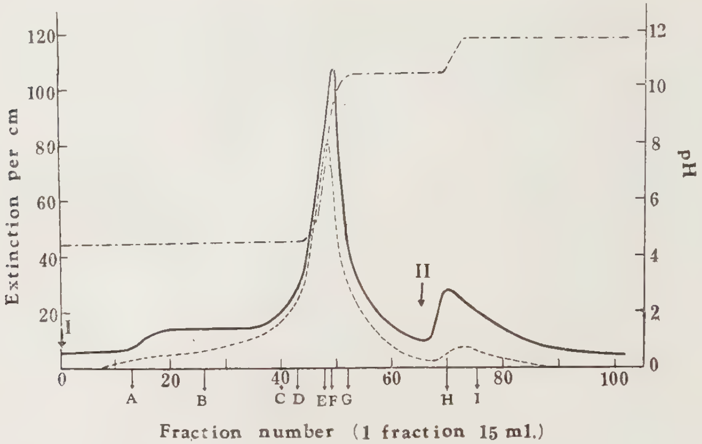

FIG. 2. Elution of DPN from Kaken cation exchange resin.

The curves show the elution process of DPN from a 3.3 × 50 cm. column of Kaken resin (60 to 100 mesh) adsorbing DPN contained in an extract from 2.5 kg. yeast with 0.5 *M* ammonium acetate buffer (pH 9.5) (I), and 0.5 *M* ammonia (II) at the flow rate of 250 ml. per hour after washing.

—— : $E^{oxi}_{260m\mu}$, - - - - - : $E^{red}_{340m\mu} \times 16.5/6.25$, -·-·-·- : pH

TABLE I

Purity of DPN in some Eluate Fractions as Indicated in Fig. 2

Fraction number	Fraction index as shown in Fig. 2	$E^{red}_{340m\mu}/E^{oxi}_{260m\mu}$
13	A	0.122
26	B	0.225
40	C	0.217
43	D	0.251
48	E	0.240
49	F	0.170
52	G	0.200
70	H	0.132
75	I	0.052
Pure DPN		0.379

with a higher purity. For instance, when the fractions, No. 40 to 47 in Fig. 2, were combined and treated as above, 0.8 g. of DPN preparation of 90 per cent purity was obtained from 10 kg. of fresh yeast.

Comparsion with Other Preparative Methods—Since the purity and the yield of DPN are variable with different starting materials, it is impossible to compare the efficiency of the present method with the others by referring only to the reported values. For this reason, we have attempted to reexamine other methods of preparation, especially the methods of LePage *et al.* (*9*) and of Clark *et al.* (*8*), using the same yeast as the starting material. The results indicate that not only these methods were laborious and time-consuming, but the yield and purity of the products were lower than these from the present method as shown in Table II.

TABLE II

Comparison of Different Methods for Preparing DPN

Method	Reported values		Reexamined values***	
	Purity	Yield*	Purity	Yield*
	%	g.	%	g.
LePage, *et al*, (*9*)	63	0.3 to 0.5	30 to 35	0.4 to 0.6
Clark, *et al.* (*8*)	85	1.0	10	2.0
Neilands, *et al.* (*10*)	80	1.2		
Animal charcoal chromatography			30	3.0
Cation exchange resin method** (Standard method)			80 to 90	1.2 to 1.5

* Yield is expressed by the values obtained from 10 kg. of pressed yeast.

** present method.

*** The results of several reexaminations according to LePage *et al.* were always near to the value shown in the Table II. The method according to Clark *et al.* was reexamined only once. The yields and purities of the product obtained by these methods were much poor compared with the values reported by them, perhaps for the differences of the starting materials.

The preparative methods of DPN with anion exchange resions recently reported by Nielands and Åkeson (*10*) and by Kornberg and Pricer (*11*) were found to be satisfactory in obtaining a very pure product (more than 95 per cent) from a partially purified DPN (about 60 per cent), but inconvenient for applying directly to the crude DPN solution, such as the extract from yeast.

Further purification by means of anion exchange chromatographies, which can easily yield the most purified DPN (more than 98 per cent) from the material prepared by cation exchange resin, will be described elsewhere.

SUMMARY

A method for the preparation of DPN, which consists of only two main steps ; chromatography on a cation exchange resin and fractionations with silver and ethanol, was described. In this purification, chromatography on the cation exchange resin—a polyphenol resin with phenoxyacetic acid and phenol as the radical—was most essential step. About 1.5 g. of white amorphous substance of 86 per cent DPN was obtained from 10 kg. of pressed bakers' yeast by this method. The method was compared with several other methods of preparing DPN.

The authors wish to express many thanks to the Oriental Yeast Ltd. and Scientific Research Institute for their kind supply of the pressed yeast and Kaken cation exchange resins respectively.

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The Journal of Biochemistry, Vol. 42, No. 4, 1955

STUDIES ON THE OXIDASE SYSTEM OF ESCHERICHIA COLI WITH SPECIAL REFERENCE TO INHIBITION OF SUCCINIC OXIDASE SYSTEM*

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(Received for publication, April 19, 1955)

Although the respiratory system of *Escherichia coli* has an atypical cytochrome system comprising the components a_1, a_2, and b_1, and the one of animal tissues or yeast has a typical cytochrome system comprising the components a, b, and c, it has been considered rather difficult to make a marked difference between the both respiratory systems, because the both systems are strongly inhibited by cyanide and carbon monoxide. In 1941, Keilin and Harpley (*1*) demonstrated the complete absence of cytochrome c and cytochrome c oxidase in *E. coli* and showed that the cytochrome a_1 or a_2 might be a component fulfilling the functions of cytochrome c oxidase. Recently, the respiratory enzyme systems of several bacteria were studied by many workers, among which Mori *et al.* (*2*) indicated the transformation of cytochrome b_4 to c in the cells of halotolerant bacteria. These facts suggest that the role of cytochrome system in cellular respiration is more dynamic and complicated than that was considered previously. As discussed by Yakushiji and Okunuki (*3, 4*), the nature of cytochrome a is very interesting, and its elucidation will contribute to the understanding of the nature and role of terminal oxidase in respiration.

The present investigation was undertaken in order to clarify the properties of oxidase systems, particularly the succinic oxidase system of *E. coli*, comparing with the typical system by the use of different inhibitors of respiration and we have obtained some knowledges about these problems. These observations show that there are marked differences in the nature of the cytochrome system, especially in the behavior towards carbon monoxide, but not in the nature of dehydrogenases. Moreover, succinic dehydrogenase is inactivated by treatment with cyanide and this differs from other kinds of dehydrogenases.

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* Aided in part by the Grant in Aid for Scientific Research from the Ministry of Education. The compendium of this article was presented at the meeting of the Symposia on Enzyme Chemistry in Osaka, July, 1952.

EXPERIMENTAL

Materials and Methods

E. coli was grown in a peptone-bouillion medium for 16 to 24 hours at 30°, collected by centrifugation, washed twice, and resuspended in distilled water. In many experiments, resting cell suspensions were mostly used, while cell-free extracts were obtained according to the method of Werkman *et al.* (*5*) and autolyzed cells were obtained according to the method of Stephenson (*6*). Oxygen consumption was measured by the Warburg manometer and activities of dehydrogenases were determined by measuring the reduction time of methylene blue added in the Thunberg tube. Alcohol and lactic dehydrogenases of bakers' yeast were prepared according to the method of Hahn *et al.* (*7*).

The details of experiments will be described in each section.

RESULTS

Oxidation of Succinate and Its Inhibitoin by Malonate—*E. coli* can oxidize some carboxylic acids involved in the Krebs' citric acid cycle such as succinate, fumarate, malate, acetate, and pyruvate, among which succinate oxidation is very active. In this organism, malonate is neither metabolizable, nor inhibitory for the oxidation of succinate. Nevertheless, the oxidation of succinate by cell-free extracts and by autolyzed cells are strongly inhibited by the addition of malonate (Fig. 1).

The same phenomena are observed in several bacteria such as *Mycobacterium tuberculosis avium* (*8*), *Pseudomonas aeruginosa* (*9*) and *Achromobacter sp.* (strain UH_3) (*10*) *etc.*, and these bacteria can adaptively decompose malonate to acetate, so that, malonate should be permeable in these bacterial cells.

Effect of Cyanide—Succinic oxidase system is strongly inhibited by the addition of a low concentration of cyanide and this inhibition is reversed by its removal from the cells.

According to Tsou (*11*), succinic dehydrogenase prepared from ox heart is inhibited irreversibly by treatment with cyanide for a long time and this inhibitory action is protected by the presence of succinate or dithionite which retains the enzyme in a reduced state. As shown in Table I, the same results have also been obtained with succinic dehydro-

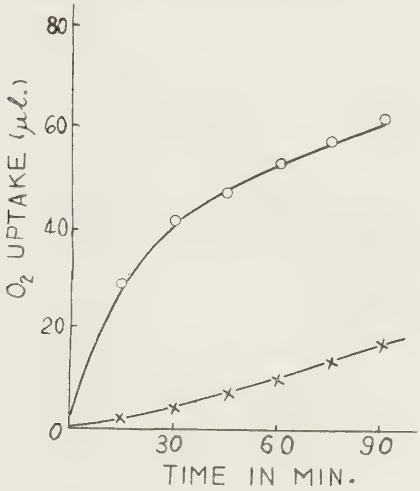

Fig. 1. Inhibition by malonate of succinic oxidase system of autolyzed cells of *E. coli.*

Experiments were carried out in the Warburg manometer. Each vessel contained ; 1.6 ml. of a suspension of autolyzed cells in the main compartment, 0.2 ml. of 0.01 *M* of substrate and of malonate in the side arm, and 0.2 ml. of 20% KOH in the center well. Total volume, 2.4 ml. Temperature, at 30°. Gas phase, in air.

—○— Succinate. —×— Succinate+malonate.

genase of *E. coli.* Furthermore, the activity of succinate oxidation by the cells, which have been treated with 0.01 *M* cyanide for 6 hours, washed several times, suspended overnight in phosphate buffer (pH 7.4), and again washed with distilled water next day in order to remove the added cyanide, is decreased by one-half as compared with non-treated cells, and when a small amount of cyanide is again added, the treated cells are further inhibited and show the oxygen uptake which is identical with that of control cells inhibited by the same amount of cyanide (Fig. 2). Any variation in the absorption spectrum of cytochrome b_1 in the visible range is not observed by these treatments. It appears to us that the treatment with cyanide is more inhibitory to succinic dehydrogenase than to oxidase system.

Similar experiments were carried out with formic and lactic dehydrogenases of *E. coli* and alcohol and lactic dehydrogenases prepared from bakers' yeast. As shown in Tables I, II, and III, the inactivation

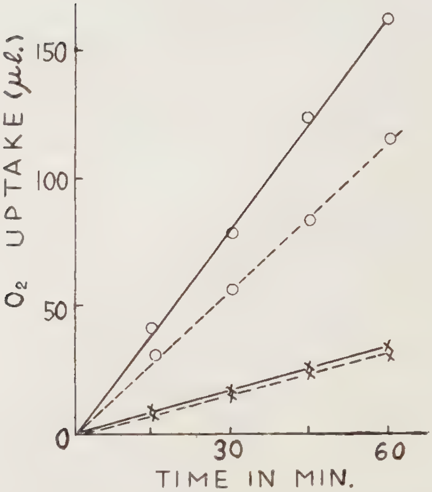

FIG. 2. Comparison of the oxidative activities of cyanide treated cells of *E. coli* with the one of non-treated cells and inhibition by cyanide of these oxidations.

Cyanide treated cells were prepared as described in text.

Each vessel contained ; 1.0 ml. of cell suspension treated with cyanide or non-treated cell suspension and 0.8 ml. of 0.1 *M* phosphate buffer (pH 7.4) in the main compartment, 0.2 ml. of 0.01 *M* succinate and of 0.001 *M* KCN in the side arm, and 0.2 ml. of 20% KOH in the center well. Other conditions were the same as in Fig. 1. The concentrations of the both bacterial suspensions were made equal by determining in a nephelometer.

—○— - - -○- - - }Succinate. ---- Cyanide treated cell.
—×— - - -×- - - }Succinate+KCN. —— Non-treated cell.

of dehydrogenases by treatment with cyanide is observed only in formic dehydrogenase which is difficult to extract because of the close combination with the cell structure as firmly as succinic dehydrogenase. In the case of formic dehydrogenase, however, this inactivation effect exists even in the presence of formate and the rate of inactivation is very rapid as compared with that of succinic dehydrogenase.

The activities of these formic and succinic dehydrogenases in living cells of *E. coli* were measured by means of Thunberg method, while manometric determinations of oxygen consumption which used methylene

TABLE I

Effect of Cyanide on Succinic and Lactic Dehydrogenases of E. coli

Conditions : 1.0 ml. of bacterial suspension (B.S.) ; 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.4) (buf.) ; 0.2 ml. of 0.01 *M* of each substrate (S) : 0.2 ml. of 0.1 *M* KCN ; and 0.2 ml. of 0.001 *M* methylene blue (MB).

Final volumes were made up to 2.8 ml. by water. After incubation for 4 hours at 37° the tubes were evacuated and kept at 38°. The contents of the tubes were mixed and reduction times of methylene blue were measured.

Tube contents		Time of reduction of MB	
Main tube	Hollow stoppers	Succinic dehydrogenase	Lactic dehydrogenase
		min.	*min.*
B.S.+buf.	MB	18	60
B.S.+buf.	MB+S	11	8
B.S.+buf.	MB+S+KCN	11	8
B.S.+buf.+KCN	MB	180	180
B.S.+buf.+KCN	MB+S	52	13
B.S.+buf.+KCN+S	MB	13	13

TABLE II

KCN-inactivation of Formic Dehydrogenase of E. coli

The experimental conditions were the same as those given in Table I except the incubation period.

Incubation period	The content		Reduction time
	Main tube	Hollow stoppers	
min.			*min.*
0	B.S.+buf.	MB	>120
	B.S.+buf.	MB+S	2
	B.S.+buf.	MB+S+KCN	2.5
30	B.S.+buf.	MB+S	2.5
	B.S.+buf.+KCN	MB+S	15
	B.S.+buf.+KCN+S	MB	9
60	B.S.+buf.	MB+S	2.5
	B.S.+buf.+KCN	MB+S	16.5
180	B.S.+buf.	MB+S	2.5
	B.S.+buf.+KCN	MB+S	45
	B.S.+buf.+KCN+S	MB	15

TABLE III

Effect of Cyanide on Lactic and Alcohol Dehydrogenases Prepared from Bakers' Yeast

Thunberg tubes contained ; 1.0 ml. of 5% enzyme solution in 0.1 *M* phosphate buffer (pH 7.4) (E), 0.2 ml. of 0.1 *M* KCN, 0.2 ml. of 0.5 *M* substrate (S), and 0.2 ml. of 0.001 *M* methylene blue (MB). Final volumes were 2.0 ml. Others were the same as in Table I.

Tube content		Reduction time of MB	
Main tube	Hollow stoppers	Lastic dehydrogenase	Alcohol dehydrogenase
		min.	*min.*
E	MB	120	9
E	MB+S	13	3.5
E	MB+S+KCN	13	3.5
E+KCN	MB	120	15
E+KCN	MB+S	13	3
E+KCN+S	MB	13	4.5

blue as an electron carrier in place of the cytochrome system inhibited by cyanide were unsuccessful. Then the autolyzed cells were used as the enzyme source for the manometric measurements of dehydrogenase activities as in the case of malonate inhibition. As shown in Fig. 3, the inhibitory action of cyanide is reversed by the added methylene blue in succinic dehydrogenase, but not in formic dehydrogenase (Fig. 4), which also confirmes the fact that the latter is inhibited very rapidly by cyanide even in the presence of formate as tested by Thunberg method.

It has also been clearly demonstrated that the inhibitory action of cyanide on lactic oxidase system of *E. coli* is somewhat reversed by the addition of 0.00017 *M* of methylene blue, which is the maximum non-toxic concentration for living cells (Fig. 5).

Therefore, methylene blue may be permeable into the cell membrane of this organism. The employment of thionine which has a higher redox-potential than methylene blue also gives similar results, from which redox-potentials may be independent in this problem. These facts will be discussed in detail in the following sections.

Inhibition by BAL (2,3-Dimercaptopropanol)—Oxidation of succinate is strongly inhibited by BAL (Fig. 6). However, this inhibition appears different from the one of BAL-sensitive factor(s) which is involved in succinic oxidase system of ox heart muscle and acts between cytochromes b and c as reported by Slater (*12*). This is based on the facts that the

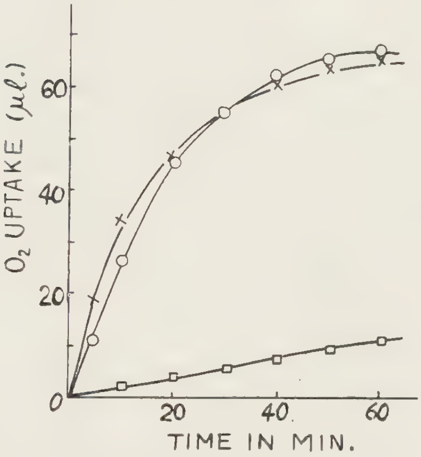

FIG. 3. Relation between succinic dehydrogenase and methylene blue.

Autolyzed cells were used as the enzyme source. Test conditions were the same as in Fig. 1 except the use of 0.001 *M* methylene blue and 0.001 *M* KCN. Total volume, 2.6 ml.

—○— Succinate+methylene blue.
—×— Succinate+methylene blue+KCN.
—□— Succinate+KCN.

oxidase system is inhibited as long as the oxidized state of BAL is being formed and inhibition of the dehydrogenase by oxidized BAL appears in about 30 minutes after the addition of BAL. The activity of succinic dehydrogenase of *E. coli* is strongly inhibited when the bacteria are treated with 0.0067 *M* of BAL for 2 minutes at room temperature and then BAL removed by several washings (Table IV). Therefore, it was unable to confirm the presence of a BAL-sensitive factor in this atypical system.

Inhibition by Carbon Monoxide—As reported by Keilin and Harpley (*1*) and by Yamagutchi (*13*), the succinic oxidase system is inhibited by carbon monoxide, but not reversed by light. However, when experiments are carried out at a low temperature of 8 to 13°, the light effect is detectable as with *Azotobacter* containing atypical cytochromes a_1 and a_2 systems reported by Negelein and Gerischer (*14*). Similar light effects at a low temperature are also observed in Azotobacter

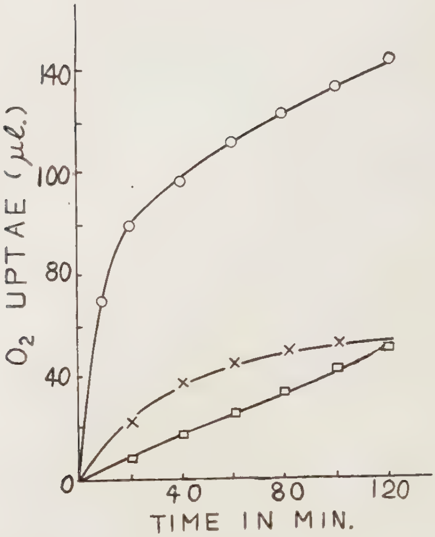

FIG. 4. Relation between formic dehydrogenase and KCN. Conditions were the same as in Fig. 3.

—○— Formate+methylene blue.
—×— Formate+methylene blue+KCN.
—□— Formate+KCN.

and Acetobacter, from which these effects should be characteristic of the atypical system (Table V). Carbon monoxide inhibition is somewhat reversed by the addition of methylene blue.

Effects of Other Materials—*E. coli* can not oxidize hydroquinone, pyrocatechol, and *p*-phenylenediamine which can reduce cytochrome c. 8-Hydroxyquinoline inhibits the oxidation of succinate by living cells, but by autolyzed cells, so that this inhibition will not be on succinic oxidase itself. Quinone has a strong inhibition, while azide has scarcely any inhibition.

DISCUSSION

Succinic oxidase system prepared from the heart was described in the scheme by Yakushiji and Okunuki (*4*) as follows:

$$\begin{tikzcd}
O_2 & \text{cyt.c} \arrow[l] \arrow[r, shift left] & \text{cyt.c}_1 \arrow[l, shift left] & x \arrow[l] & \text{cyt.b} \arrow[l] & & \text{succinate} \arrow[ll] \\
\text{cyt. c-oxidase} \arrow[u] & \text{cyt.a} \arrow[ur, dashed] & & & & \text{succinic dehydrogenase} \arrow[u] &
\end{tikzcd}$$

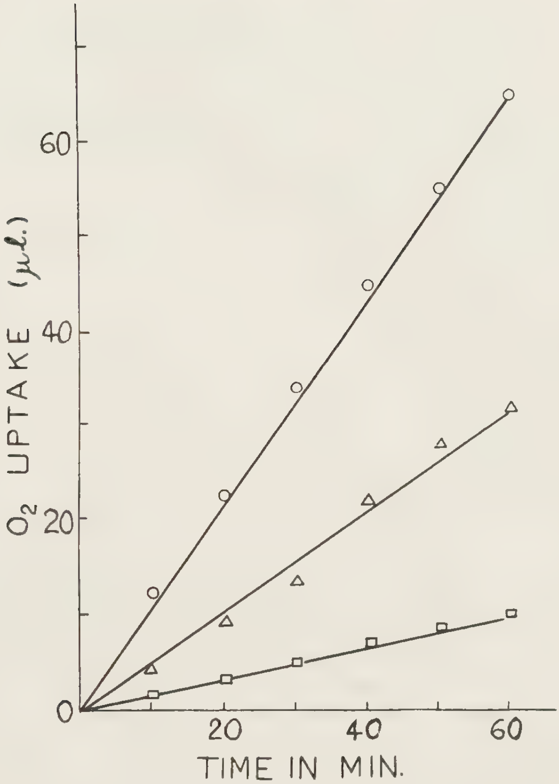

FIG. 5. Relation between lactic dehydrogenase system in living cells of *E. coli* and methylene blue.

Conditions were the same as in Fig. 2, except the use of 0.00017 *M* methylene blue and 0.001 *M* KCN. Total volume, 2.6 ml.

—○— Lactate+methylene blue.
—△— Lactate+methylene blue+KCN.
—□— Lactate+KCN.

It is impossible to regard cytochrome a as cytochrome oxidase because cytochrome a isolated from the heart muscle is a non-autoxidizable component and cytochrome oxidase can only oxidize cytochrome c but not cytochrome a. On the other hand, according to the studies of cytochrome oxidase by Melnick (*15*), and of absorption spectra of cytochrome a and its CO-complex by Okunuki and Yakushiji (*4*)

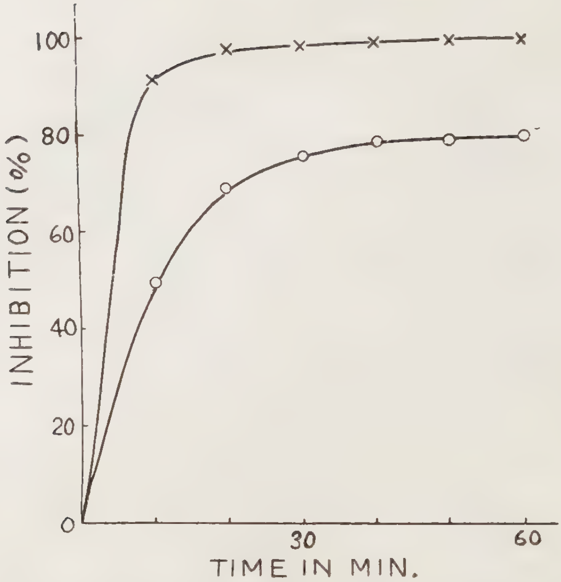

FIG. 6. Inhibition by BAL on succinic oxidase system of *E. coli*. The living cells and cell-free extracts were used. Reaction mixture contained 0.0065 *M* BAL.

—×— Cell-free extracts.

—○— Living cells.

TABLE IV

Effect of BAL on Succinic Dehydrogenase of E. coli

The activities of succinic dehydrogenase treated with and without 0.0067 *M* BAL as described in text were measured by Thunberg tube as shown in Table I.

	Succinate	Reduction time of MB
		min.
Non-treated cell	—	>120
”	+	21
BAL-treated cell	—	>120
”	+	>120

and by Okunuki and Sekuzu (*16*), the Soret band of CO- complex of cytochrome a_3, *i.e.*, cytochrome oxidase described by Keilin and

TABLE V

Relation between the Rate of CO-inhibition of Oxidase Systems and Its Reversal by Light at Various Temperatures

Bacteria	Substrate	Temp.	Inhibition rate by Co		Recovery of inhibition
			In dark	In light	
			%	%	%
E. coli	Succinate	30°	72	69	4.1
			70	68	2.8
		8°	61	41	32.7
			58	40	31.0
Azotobact. chroococcum	Glucose	30°	55	52	5.4
		8°	48	39	18.7
Acetobact. sp.	Ethyl alcohol	30°	76	68	10.5
			72	60	16.6
		13°	69	17	75.4
			65	15	76.8

Hartree (*17*), does coincide with the Soret band of CO-complex of cytochrome a. Therefore, the scheme of cytochrome system introduced by Keilin and Hartree is misleading in the point that cytochrome a is directly oxidized by cytochrome a_3.

In succinic oxidase system of *E. coli* with atypical cytochromes, cytochrome b_1 and its oxidase are present instead of cytochrome c and cytochrome c oxidase involved in a typical system. Since it was certified that the succinic dehydrogenase of *E. coli* is similar to that of animal tissues and the presence of a BAL-sensitive factor is indistinct, the study of cytochrome b_1 is very interesting, but unfortunately, no knowledge about this is existent.

Cytochrome a_1 or a_2 may fulfill the function of cytochrome b_1 oxidase or there may be an unknown component which might be called cytochrome b_1 oxidase. In either case, this component is strongly bound with carbon monoxide and its binding is sensitive to light at a low temperature of 8 to 13°, but not at 30°. The investigations of these cytochrome systems belonging to the family of heme of color mixed will throw light on the important mechanism of the action of cytochromes taking the nearest position to oxygen, namely of terminal oxidase.

Since the activity of succinic oxidase system of the living cells measured in manometer suffers no inhibition by malonate and takes less reduction of methylene blue, the above reagents seem to be screened by some membrane, which may be not the plasmamembrane, but the membrane of particles such as mitochondria in cytoplasm. This is suggested by the following facts:

a) As described in results, malonate is unable to inhibit the oxidation of succinate by several bacteria and has a strong inhibition against the enzyme system liberated from these cells.

b) Lactic dehydrogenase localized in cytoplasm can rapidly reduce the added methyene blue in the presence of cyanide, while succinic and formic dehydrogenases combined closely with cell structures can not.

Succinic dehydrogenase is inactivated by treatment with cyanide, but not in the presence of succinate. Formic dehydrogenase is also inactivated by cyanide even in the presence of formate. For that reason, the nature of formic dehydrogenase in *E. coli* which needs no co-enzyme (*18*) is similar to that of higher plants which require co-enzymes (*19*). However, lactic and alcohol dehydrogenase are cyanide-insensitive, which differ from the enzymes mentioned above.

SUMMARY

The nature of succinic dehydrogenase of *E. coli* has been demonstrated to be similar to that of animal tissues so far experimented, particularly by treatment with cyanide it is irreversibly inhibited and unaffected in the presence of succinate. Contrary to the succinic dehydrogenase, formic dehydrogenase is rapidly inactivated even in the presence of formate by a short contact with cyanide. Lactic dehydrogenase of *E. coli* and lactic and alcohol dehydrogenases prepared from yeast are not inhibited by this treatment.

Succinic oxidase system of *E. coli* is different from that of the typical system, for it has a strong affinity to carbon monoxide.

The significance of the results to the localization of succinic oxidase system in cells and the mechanism of atypical ctyochrome system in cellular respiration is discussed.

We are most grateful to Dr. J. Hirade and to the Daiichi Seiyaku Co. for their gifts of BAL.

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The Journal of Biochemistry, Vol. 42, No. 4, 1955

STUDIES ON CHROMOPROTEINS IN JAPANESE NORI (PORPHYRA TENERA)

I. A NEW METHOD FOR THE CRYSTALLIZATION OF PHYCOERYTHRIN AND PHYCOCYANIN

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(Received for publication, April 20, 1955)

Two water-soluble pigments have been isolated from various seaweeds. Kützing (*1*) gave the name of phycoerythrin to the red pigment in *Phodophycea* (red algae) and phycocyanin to the blue pigment in *Cyanophycea* (blue green algae). Molish (*2*) elucidated that they are chromoproteins and can be crystallized from its ammonium sulfate solution. Further considerable advances have been made in the chemistry of these chromoproteins particularly through the works of Kylin (*3*), Kitasato (*4*) and Lemberg. (*5*, *6*, *7*)

It is interesting that these chromoproteins seem to be analogous to haemoglobin in structure and physiological function.

In the previous studies, crystallization of the chromoproteins was achieved by fractional precipitation with ammonium sulfate. This process of purification was laborious and poor in yield even from the best available source of Japanese " Nori ", *Porphyra tenera*, and especially the purification of phycocyanin was found to be difficult.

Tiselius (*8*) reported that he was unable to purify the pigments by mechanical precipitation with ammonium sulfate but their complete separation could be made with success using electrophoretic analysis.

A chromatographical method for the separation of the algal chromoproteins has recently been provided by Swingle and Tiselius (*9*) which involves adsorption of the pigments on tricalcium phosphate columns.

The present author has now found that an abundance of mucous substance named phycocolloid by Tseng (*11*) in the extracts from Nori disturbed the separation and the crystallization of these pigments. The author applied rivanol (2-ethoxy-6,9-diaminoacridinum lactate), which had been used by Hayashi (*12*) and Akabori (*13*) in the purification of taka-amylase, to the removal of phycocolloid and to the separation of two chromoproteins and succeeded in the purification of phycoerythrin and phycocyanin.

EXPERIMENTAL

The preparation of two crystalline proteins, phycoerythrin and phycocyanin, from Japanese Nori (which is generally called Asakusa-Nori) is outlined schematically in Table I. Commercial products of dried Nori made along the coast of Eastern Japan, which consist chiefly of *Porphyra tenera* tenera, were used as a source of both pigments.

Purification and Fractionation of Phycoerythrin and Phycocyanin—To 1 kg. of dried material. 20 litres of water were added, and after being kept in the dark for 3 or 4 days, brownish purple extracts were obtained. The extracts were pressed out using a cotton bag, and $1M$ calcium acetate solution was added to it up to the final concentration of $0.1\ M$. After 30 minutes, it was filtered through celite, and 400 g. of ammonium sulfate was added per liter of the filtrate. The chromoproteins precipitated were dissolved in 10 liter of water, dialyzed in cellophane bags for 2 to 3 days, 100 ml. of 1 per cent rivanol solution were added to the dialyzed solution, and the precipitate was filtered off after 30 minutes.

The *Filtrate II* was mixed with 300 ml. of 1 per cent rivanol solution, wherepuon phycoerythrin-rivanol symplex precipitated which was washed with $M/2$ NaH_2PO_4 and then with water for the removal of rivanol. By dissolving the precipitate in 10 per cent solution of ammonium sulfate, phycoerythrin solution was obtained. On adding Japanese acid clay to the *Filtrate IV*, extracts with NaH_2PO_4, brown colored materials and rivanol were absorbed on the clay, and when the filtrate became purple-blue, 200 g. of ammonium sulfate per liter were added to the solution and pH was ajusted to pH 6.0, where phycocyanin was precipitated, while phycoerythrin remained in the solution. The latter was precipitated by addition of further 100 g. of ammonium sulfate to the filtrate.

Japanese acid clay was added to the *Filtrate III* to remove rivanol. To the filtered blue solution 4 kg. of ammonium sulfate were added in order to precipitate phycocyanin.

The precipitate of phycocyanin was dissolved in water, dialyzed followed by centrifugation, and then to the supernatant was added 1/4 volume of saturated ammonium sulfate solution. The phycoerythrin solution was treated in the same way as in the case of phycocyanin, except the addition of 1/3 volume of ammonium sulfate solution instead of 1/4. By keeping both solutions in ice-box an overnight, well defined crystals of chromoproteins were obtained. The photomicrographs of the crystals are shown in Fig. 1.

The pressed cake of Nori (*Fraction II*), was stirred with 5 *l.* of water, kept standing for two weeks below 10° in the presence of a small quantity of toluene, and then was treated as described above. As chromoproteins are sensitive to light, direct sun light or illumination has been cautiously avoided throughout the treatments.

TABLE I

Scheme for the Crystallization of Phycocyanin and Phycoerythrin

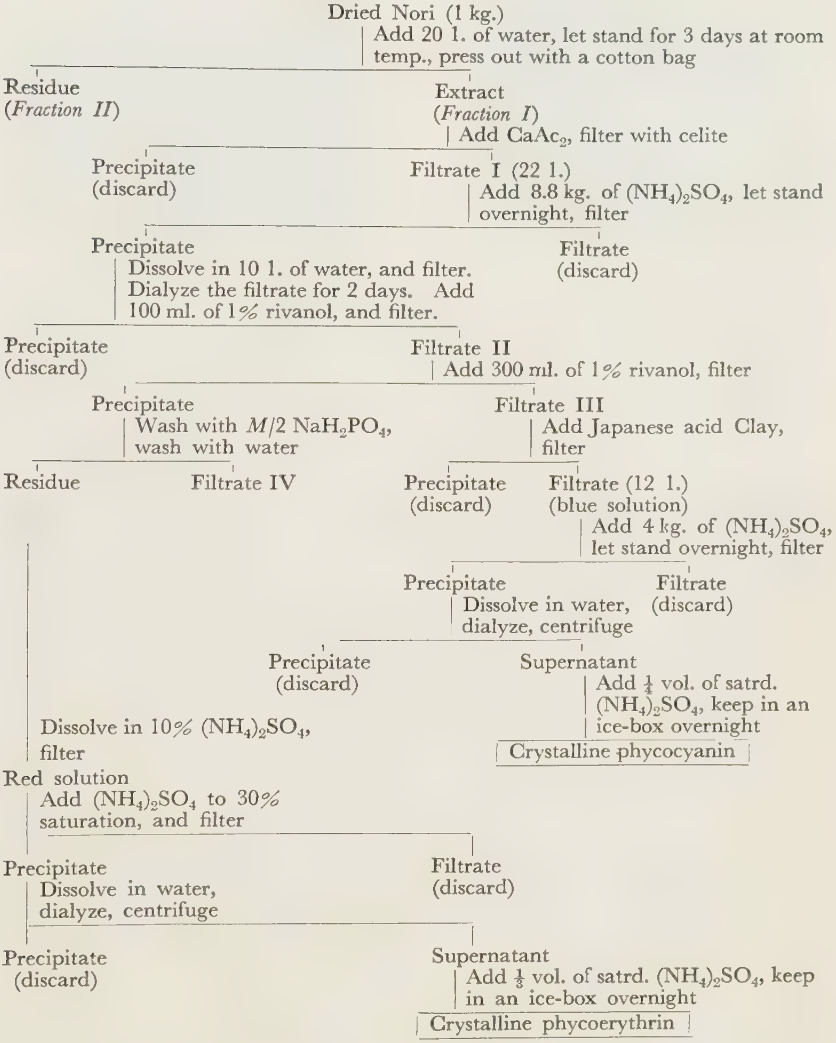
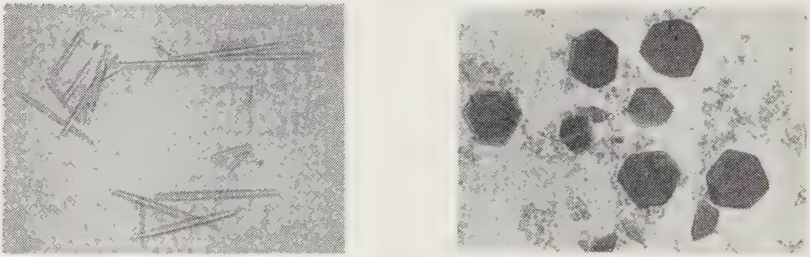

Dried Nori (1 kg.)
Add 20 l. of water, let stand for 3 days at room temp., press out with a cotton bag
Residue (*Fraction II*)
Extract (*Fraction I*)
Add $CaAc_2$, filter with celite
Precipitate (discard)
Filtrate I (22 l.)
Add 8.8 kg. of $(NH_4)_2SO_4$, let stand overnight, filter
Precipitate
Dissolve in 10 l. of water, and filter. Dialyze the filtrate for 2 days. Add 100 ml. of 1% rivanol, and filter.
Filtrate (discard)
Precipitate (discard)
Filtrate II
Add 300 ml. of 1% rivanol, filter
Precipitate
Wash with $M/2$ NaH_2PO_4, wash with water
Filtrate III
Add Japanese acid Clay, filter
Residue
Filtrate IV
Precipitate (discard)
Filtrate (12 l.) (blue solution)
Add 4 kg. of $(NH_4)_2SO_4$, let stand overnight, filter
Precipitate
Dissolve in water, dialyze, centrifuge
Filtrate (discard)
Precipitate (discard)
Supernatant
Add $\frac{1}{4}$ vol. of satrd. $(NH_4)_2SO_4$, keep in an ice-box overnight
Crystalline phycocyanin
Dissolve in 10% $(NH_4)_2SO_4$, filter
Red solution
Add $(NH_4)_2SO_4$ to 30% saturation, and filter
Precipitate
Dissolve in water, dialyze, centrifuge
Filtrate (discard)
Precipitate (discard)
Supernatant
Add $\frac{1}{3}$ vol. of satrd. $(NH_4)_2SO_4$, keep in an ice-box overnight
Crystalline phycoerythrin

Fig. 1. Photomicrographs of crystalline phycoerythrin and phcocyanin (*Porphyra tenera*)



Phycoerythrin (×800) Phycocyanin (×800)

Properties—Both chromoproteins crystallize from their water solution by addition of ammonium sulfate, but not by any of organic solvents such as alcohol and acetone.

As can be seen from the photomicograph in Fig. 1, phycoerythrin crystallized in small needles or prisms ; phycocyanin in needles or platelets. The aqueous solution of phycoerythrin is bright red in color with yellow fluorescence, that of phycocyanin being blue in color with red flucoresence as observed by many previous authors.

Preparations of both chromaproteins obtained by the author seem to be electrophoretically pure, as determined by the method of McDonald (*14*) in the phosphat buffer of pH 6.4, 6.8 and 7.2. Phycoerythrin moved to the cathode faster than phycocyanin at these pH. Isoelectric points of phycocyanin and phycoerythrin were reported as pH 4.3 and 4.5 by Lemberg (*5*) and as pH 4.25 and 4.85 by Tiselius (*8*).

After repeated recrystallization, phycoerythrin shows a positive Molisch's reaction and seems to be a glucoprotein. The results of elemental analysis of phycoerythrin and phycocyanin, which were recrystallized from ammonium sultate solution several times and dialyzed seven days at 5° are given in Table II.

Absorption Spectra—Absorption spectra of the purified physoerythrin and phycocyanin

TABLE II

Analysis of Phycoerythrin and Phycocyanin (Porphyra tenera)

Phycoerythrin (%)

C	H	N	S	Ash	References
50.80	7.99	15.13	2.06	1.06	(by the author)
50.87	7.04	15.34	1.76	—	(by Kitasato (*4*))
Phycocyanin (%)					
51.01	6.82	15.81	1.12	1.52	(by the author)
50.60	6.90	15.76	1.69	—	(by Kitasato (*4*))

in 15 μM concentration in 0.05 M phosphate buffer (pH 6.8) have been measured in the visible and ultra-violet region by a Beckman spectrophotometer. The spectral curves are shown in Figs. 2 and 3. Remarkable is the difference in the spectra of the

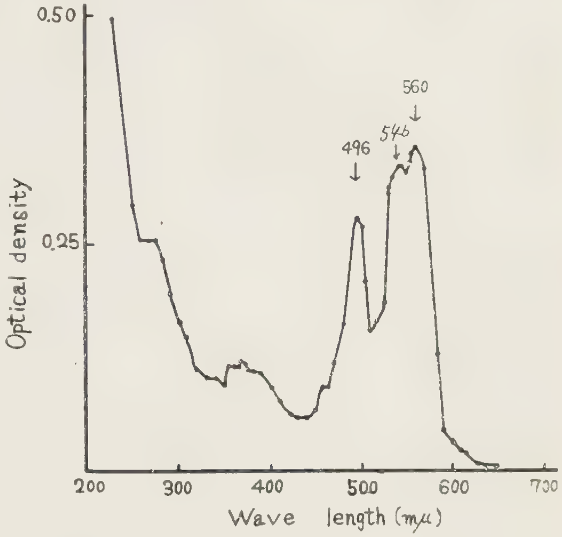


Fig. 2. Spectral absorption curves of phycoerythrin (15 μM) in phosphate buffer at pH. 6.8.

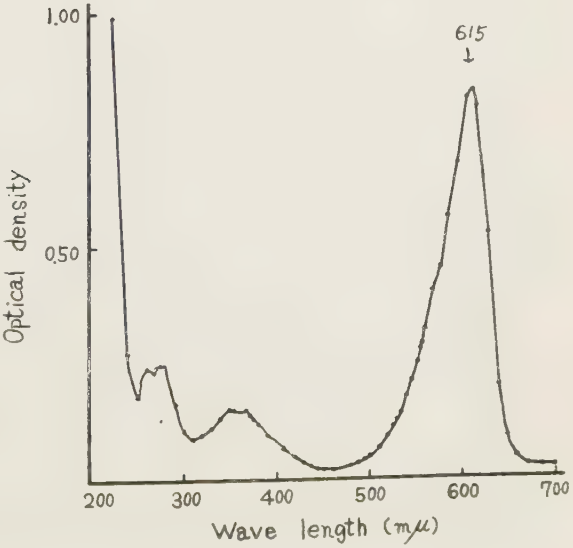


Fig. 3. Spectral absorption curves of phycocyanin (15 μM) in phosphate buffer at pH. 6.8

TABLE III

Absorption Maxima of Phycoerythrin and Phycocyanin

(R and C denote aglal divisions *Rhodophyceae* and *Cyanophyceae*, respectively.)

Phycoerythrin

Alga	Authors	Absorption maxima (mμ)			pH
Porphyra tenera (R)	Fujiwara	560	546	496	6.8
	Tiselius (*8*)	560	546	496	6.8
	Kitasato (*4*)	560	526	495	—
	Svedberg (*15*)	555	534	496	6.8
Porphyra pseudolinearis (R)	Takagi (*16*)	555	533	495	6.8
Porphyra perforata (R)	Haxo *et al.* (*10*)	562	—	497	6–7
Ceramium rubrum (R)	Svedberg (*15*)	555	529	496	6.8
Rhodymenia pacifica (R)	Haxo *et al.* (*10*)	564	537	497	6–7
Phormidium ectocarpi (C)	Haxo *et al.* (*10*)	565	542.5	—	6–7
Phormidium persicinum (C)	Haxo *et al.* (*10*)	560	—	—	6–7
Phycocyanin					
Porphyra tenera (R)	Fujiwara	615	—		6.8
	Tiselius (*8*)	610	552		6.8
	Kisatato (*4*)	610	546		—
	Svedberg (*15*)	610	552		6.8
Porphyra pseudolinearis (R)	Takagi (*16*)	610	552		6.8
Porphyra perforata (R)	Haxo *et al.* (*10*)	617	555		6–7
Ceramium rubrum (R)	Svedberg (*15*)	621	546		6.8
Aphanizomenon flos aquae (C)	Svedberg (*15*)	615			6.8
Lyngbya Lagerheimii (C)	Haxo *et al.* (*10*)	650			6–7

visible range between the two chromoproteins. Phycocyanin shows strong absorption at 615 mμ and weak absorptions from 340 to 366 mμ and from 260 to 275 mμ, while phycoerythrin shows strong absorptions at 496, 546 and 560 mμ, and weak absorptions from 270 to 274 mμ and from 350 to 366 mμ. Three-peaked phycoerythrin and two-peaked phycocyanin on the visible absorption have generally been observed in *Porphyra tenera*. (*4,8,15*) These results indicate that phycoerythrin gives absorption maxima similar to that of *Phodophyceae*, but phycocyanin has one absorption maximum at 615 mμ which is identical with that of *Cyanophyceae* (*10,15*).

The values of absorption maxima of these chromaproteins reported by the previous investigators are given in Table III.

SUMMARY

Crystalline phycoerythrin and phycocyanin were easily prepared by the use of rivanol (2-ethoxy-6,9-diaminoacridinum lactate from the dried Japanewe Nori, *Porphyra tenera*. These so chromoproteins were confirmed to be electrophoretically pure. The absorption spectra of phycoerytlrin and phycocyanin were observed and compared with those reported before.

The author wishes to express her gratefulness to Prof. S. Akabori for his encouragement and kind guidance throughout this work.

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The Journal of Biochemistry, Vol. 42, No. 4, 1955

STUDIES ON PTERIDINES

VII. " ICHTYOPTERIN ", ITS NONIDENTITY WITH ISOXANTHOPTERYL-6-ACETIC ACID

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(Received for publication, April 20, 1955)

A substance with a purple fluorescence has previously been isolated from the scales of Japanese carps (*1*). This substance is composed of three components, the Rf values being 0.12, 0.35, and 0.39 in BuOH : AcOH :H_2O (4 :1 :1 by volume) at 20° (Toyo Filter Paper No. 50) ; the first two are identical with *iso*xanthopteryl-6-carboxylic acid (VI) and *iso*xanthopterin (II), respectively, while the last pterin (I) has been shown to be identical with " ichtyopterin " through direct comparison with material generously sent from Dr. Hüttel. Namely, the scales of *Leuciscus rutilus* (*2*) which were kindly sent by Dr. Hüttel were subjected to an identical extraction, and though a complete purification was difficult on account of the small quantity, it has been confirmed that the German material is likewise composed of three substances, *iso*xanthopteryl-6-carboxylic acid, *iso*xanthopterin, and " ichtyopterin ".

Based on the Rf values and UV spectra R. Tschesche and F. Korte (*3*) have reported that " ichtyopterin " is identical with *iso*xanthopteryl-6-acetic acid (VII). However, the following points are inconsistent with their conclusion.

i) Though the Rf values of ichtyopterin and synthetic *iso*xanthopteryl-6-acetic acid were identical in 3 per cent NH_4Cl, the Rf values apparently differed in BuOH :AcOH :H_2O (4 :1 :1), and in 4 per cent sodium citrate.

ii) While I was converted into *iso*xanthopterin by Al-Hg in alkaline media, VII remained unaffected.

iii) *Iso*xanthopteryl derivatives having 6-CH_2COOH, -$CH(CH_3)$-$COOH$, and -$CH_2CHCOOH$, CH_2CH_2COOH side chaines were readily esterified by dry HCl and MeOH (*4*) ; " ichtyopterin ", on the other hand, was unesterified.

iv) Though VII was readily decarboxylated to II by boiling in 1 N HCl for a few minutes, " ichtyopterin " remained unchanged. If " ichtyopterin " is VII as proposed by R. Tschesche and F. Korte, indeed the substance isolated should be III since Hüttel's method employs the treatment with boiling 1 N HCl.

The behavior of synthetic 6-substituted *iso*xanthopteryl derivatives towards Al-Hg and MnO_2 (*5*) together with other caracteristics, are summarized in Table I. The compounds responding in a manner similar to ichtyopterin in the Al-Hg and MnO_2 treatments were XI, XII, and XV. However, XI differed from ichtyopterin in the fact that it was readily converted into III upon heating in alkaline media ; ichtyopterin was stable (this latter observation is against that of R. Tschesche and F. Korte (*3*)), XV also differed from ichtyopterin in respect to the Rf value. Though XII also is unidentical with ichtyopterin, the chemical behaviors to MnO_2 oxidation, Al-Hg reduction and esterification and

TABLE I

The Rf Values of Ichtyopterin and iso*Xanthopterin Derivatives, and Their Behavior to Chemical Treatments*

No.	Side chains of 6-substituted *iso*-xanthopterins	Rf values*		Al-Hg reduction (products)	MnO_2 oxidation (products)	Heat with 1N HCl (procucts)
		a	b			
I	Ichtyopterin	0.39	0.63	-H	-COOH	No change
II	-H	0.35	0.40	No change	No change	»
III	$-CH_3$	0.46	0.37	»	»	»
IV	$-C_2H_5$	0.68	0.43	»	»	»
V	$-C_3H_7$	0.86	0.48	»	»	»
VI	-COOH	0.12	0.68	-H	»	»
VII	$-CH_2COOH$	0.32	0.73	No change	-COOH	$-CH_3$
VIII	$-CH(CH_3)COOH$	0.50	0.78	»	-COOH	$-C_2H_5$
IX	$-CH(C_2H_5)COOH$	0.73	0.82	»	-COOH	$-C_3H_7$
X	$-CH_2CH_2COOH$	0.33	0.62	»	No change	No change
XI	$-CH_2COCH_3$	0.51	0.56	-H	-COOH	»
XII	$-CH(OH)CH_3$	0.43	0.33	-H	-COOH	»
XIII	$-CH_2CH(OH)CH_3$	0.59	0.55	No change	No change	»
XIV	$-CH_2OCH_3$	0.51	0.52	$-CH_3$	»	»
XV	$-CH_2NH_2$	0.15	0.39	-H	-COOH	—

* Solvent : a, Butanol, acetic acid, water (4:1:1) ; b, 4% Na citrate solution.

also Rf value correlations suggest that " ichtyopterin " is presumable *iso*xanthopteryl compound with a 6-side chain possessing more than one hydroxyl group, one of them being located on the α-carbon atom.

EXPERIMENTAL

Separation of ichtyopterin, iso*xanthopterin, and* iso*xanthopteryl-6-carboxylic acid from carp scales*—4 *l.* of 3 per cent NaOH was added to about 8 *l.* of wet scales and after incubating at 37° for 2 days the scales were filtered. The filtrate was warmed to 70°, acidified with acetic acid, and centrifuged. The fluorescent substance was adsorbed on 40 g. of carbon, and subsequently eluted more than eight times with 150 ml. of 3 per cent NaOH containing 10 per cent ethanol ; this carbon adsorption and elution procedures were repeated twice. The acidified eluate was adsorbed on a chromatographic tube of 3 g. of carbon, and eluted with alkaline ethanol, when most of the fluorescent substance could be eluted with a small amount of solvent ; this procedure was useful for the concentration of pterins. The eluate was saturated with CO_2 and centrifuged. The precipitate was dissolved in an equal volume of 30 per cent NaOH, and freezed in an ice-box upon which crystals of guanine were obtained. The filtrate was diluted to 100 ml. and concentrated by the said chromatographic methods. The concentrate was added to 50 ml. of boiling $2N$ HCl and centrifuged while hot. After purification of the precipitate through the NaOH-HCl precipitation procedure, 0.95 mg. of *iso*xanthopteryl-6-carboxylic acid was obtained. The supernatant was stored in an ice-box for one night and the crystals of guanine hydrochloride were filtered. After addition of 3 g. of sodium acetate, the filtrate was passed through an alumina column, when *iso*xanthopterin was adsorbed on the top and ichtyopterin under it, only ichtyopterin was eluted with 3 per cent HCl containing 5 per cent acetic acid; *iso*xanthopterin was eluted with 1 per cent NaOH. The respective eluates were purified and concentrated by carbon chromatography. The concentrated solution were acidified with acetic acid, and stored in an ice-box. Finally, the precipitates were collected with a centrifuge and washed with water and methanol, thus giving 2.34 mg. of ichytopterin and 2.13 mg. of *iso*xanthopterin. The ichtyopterin was still contaminated with a small amount of guanine.

Al-Hg Reduction—One end of an aluminum wire (30×0.5 mm.) was amalgamated by conventional methods. This end was dipped into a 0.02 ml. pterin solution contained in a micro test tube, when hydrogen was generated. The reaction ended after 10 minutes. The aluminum hydroxide precipitate was centrifuged and the supernatant was employed for paper chromatography.

MnO_2 Oxidation—About 0.05 ml. of pterin solution and small amounts of MnO_2 were sealed in a micro tube, and the mixture was heated for one hour at 120° with occasional shaking. The MnO_2 precipitate was centrifuged and the solution was submitted to paper chromatography.

SUMMARY

Three kinds of pterins were extracted from the scales of common Japanese carps (*Cyprinus carpio* and *C. auratus*) and were shown, through direct comparison, to be identical with the three components contained in *Leuciscus rutilus*. The three pterins are *iso*xanthopterin, *iso*xanthopteryl-6-carboxylic acid, and " ichtyopterin ". Contrary to the observations made by German authors, our findings provide conclusive evidence that " ichtyopterin " is unidentical with *iso*xanthopteryl-6-acetic acid. " Ichtyopterin " is an *iso*xanthopteryl derivate possessing presumable an α-hydroxylated side chain.

The authors are greatly indebted to Prof. Fuijo Egami and Koji Nakanishi for valuable advices. A part of the cost of this research was defrayed from the Scientific Research Encouragement Grant from the Ministry of Education.

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The Journal of Biochemistry, Vol. 42, No. 4, 1955

VERDOHEMOGLOBIN, AN IMMEDIATE PRECURSOR OF BILIVERDIN IN THE MODEL REACTION OF HEMOGLOBIN BREAKDOWN IN VITRO*

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(Received for publication, April 21, 1955)

It has been known for many years that hemoglobin and pyridine hemochrome are decomposed aerobically in the presence of ascorbic acid with the formation of a biliverdin precursor, which transforms into biliverdin on addition of dilute HCl. The mentioned reaction systems have drawn attention of several investigators as the *in vitro* model reaction of physiological hemoglobin breakdown (*1*,*2*,*3*). In the case of pyridine hemochrome system the yield of biliverdin is much higher than in hemoglobin system. More detailed studies on the reaction sequence of the pyridine hemochrome system have revealed that the chemical process occurred through two stages, consecutively giving a green verdohemochrome as the biliverdin precursor (*4*,*5*). The reaction sequence has been determined to be as follows:

Pyridine hemochrome→630-substance→verdohemochrome

In the reaction with hemoglobin, however, the biliverdin precursor has been assumed to be choleglobin since the studies of Lemberg (*6*), though it has not yet been experimentally demonstrated that the chemical process follows in a sequence similar to that with pyridine hemochrome. More recently, Lemberg (*7*) has suggested that the immediate precursor of biliverdin formed in this reaction system may not be choleheme, but rather a product of more oxidized form chemically corresponding to verdoheme.

The occurrence of an intermediate corresponding to verdoheme in the reaction system of hemoglobin has not yet been experimentally demonstrated.

With the supposition that the chemical process leading to the immediate biliverdin precursor in both series of reaction system starting from hemoglobin and from pyridine hemochrome may essentially be the same, the authors have undertaken the reinvestigation of the process of choleglobin formation by use of hemoglobin and of myoglobin. As a matter of fact, the present authors have proved a new compound, an immediate precursor of biliverdin in the final stage of this reaction system corresponding to the well known verdoheme. The new compound was characterized by its absorption in longer wave length, namely at about 700–800 mμ. The absorption at these wave lengths has hitherto never been described by other investigators.

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* This makes an essential part of the lecture read by K. Kaziro before the Symposium on biological pigments at the 26th General Meeting of the Japanese Biochemical Society held on April 1954 at Sendai.

The following is the description of our experimental findings on this problem so far obtained. Based on the results obtained, a reaction scheme of the present reaction process will be presented.

EXPERIMENTAL

I. Experiments with Hemoglobin

Absorption Change during the Reaction Process—The material used was crystalline HbO_2 of horse prepared by the method of Suzuki *et al.* (*8*). The reaction solution of the composition described in Fig. 1 was separately taken into several beakers of adequate capacity and kept in a thermostat at 37°. After definite time intervals, one of the beakers was taken out, the reaction was stopped by addition of 10 ml. of 1 per cent NaOH, reduced by the addition of small amount of powdered Na_2SO_4 and the absorption was measured spectrophotometrically by the using of the apparatus of Hitachi & Co. The absorption figures are shown in Fig. 1. In earlier stages of the reaction, the absorption at 558 mμ and 527 mμ, namely of a lkali denatured globin protohemochrome, diminished and in turn there arose another absorption at 618 mμ which is of the alkali denatured globin cholehemochrome.

After the reaction time lasting over two hours, however, there arose a diffuse absorption at wave lengths longer than 700 mμ.

Extraction of Biliverdin—Extraction of biliverdin from the reaction solution was performed by the method described by Lemberg (*6*), and Engel (*9*). Into the reaction solution after each reaction time, glacial acetic acid was added as much as to give a clear solution, from which heme was extracted in ether. Biliverdin could be extracted with diluted HCl from this ether layer. Biliverdin-HCl gives an absorption maximum at 665 mμ (ε_{mM}=28.6) (*10*). The yield of biliverdin thus obtained could then be calculated from ε_{665} estimated. The yield of biliverdin increased with reaction time. Maximum yield which was about 12 per cent of total heme was recorded after 6 hours. Cholehemochrome, a green precipitate, which was formed during the reaction was collected and washed with water to remove the water soluble substances. The precipitate was examined for the existence of biliverdin, but with negative results though its heme part is undoubtedly choleheme.

Now, the reaction solution in two beakers after sufficiently longer reaction time was

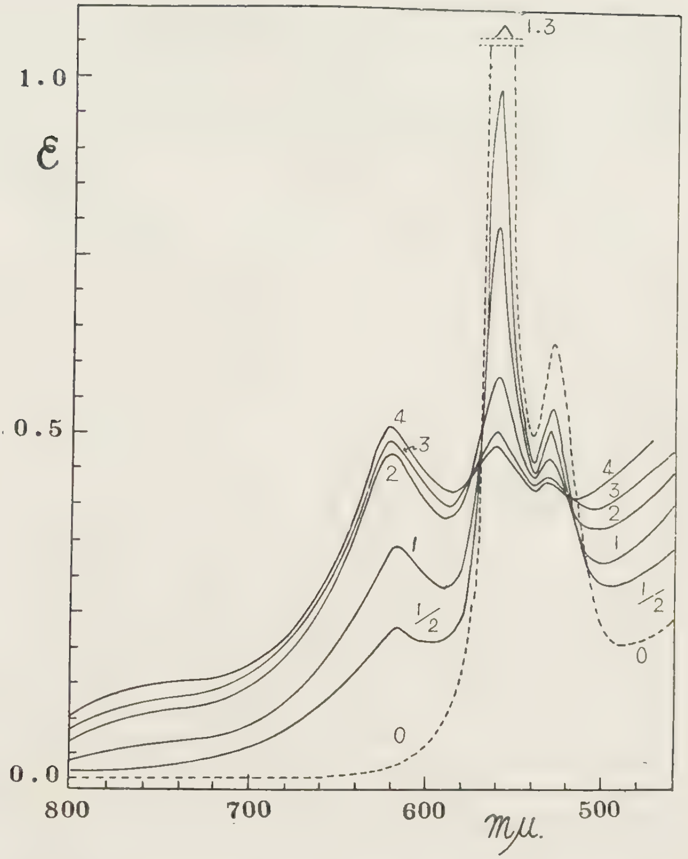


Fig. 1. Absorption change during the reaction process with hemoglobin.

2.5 ml.......3×10^{-4} *M* (heme) HbO_2

2.0 ml.......*M*/15 phosphate buffer. (pH 7.0)

0.5 ml.......0.1 *M* ascorbic acid (neutralized with NaOH) T : 37°

Absorption of the reaction solution was measured after the addition of 10 ml. of 1% NaOH and of a small amount of powdered $Na_2S_2O_4$.

investigated in the following way. To one of the beakers containing reaction solution, NaOH and $Na_2S_2O_4$ were added and was examined spectrophotometrically. From the same reaction solution in another beaker, biliverdin was extracted with acid ether, the remaining water layer was made alkaline by addition of NaOH, reduced with $Na_2S_2O_4$

and examined spectrophotometrically. The absorption figures of these two samples were compared with each other after the correction of their dilution (Fig. 2). As shown by the comparison of these two figures (Fig. 2, I and II), the absorption maximum at 618 mμ was essentially restored after biliverdin extraction, while that at 700–800 mμ completely disappeared. Thus, it seems to be without doubt that the biliverdin precursor was contained in the reaction solution as that part which showed the absorption at 700–800 mμ. Additionally it was revealed that free biliverdin, though in much lower concentrations, was formed during the reaction. This was proved as follows. The reaction solution after each time intervals was shaken with amylalcohol without acid treatment, the amylalcohol layer was separated by centrifugation and this was examined spectrophotometrically after the addition of glacial acetic acid. Absorption of biliverdin could be clearly demonstrated in this acid amylalcohol layer, though slightly, after 4 hours of reaction time.

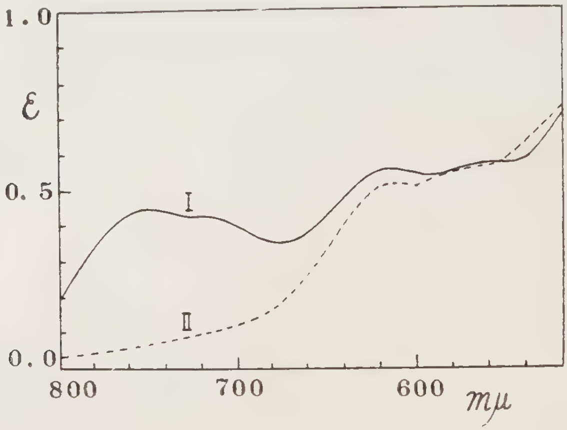


FIG. 2. Comparison of the absorption figures of the two samples before and after the treatment for the biliverdin extraction.

Full line : before the treatment for biliverdin extraction.

Broken line : after that the biliverdin was extracted.

II. Reaction with Myoglobin

Absorption Change during the Course of the Reaction Process—Crystalline myoglobin was prepared from heart muscle of horse by the method devised in our laboratory (*11*). The experiment was conducted in a manner similar to those with hemoglobin. As the protein part of myoglobin is much more resistant against denaturation, green precipitate was not formed during the reaction, and further, the treatment with NaOH and $Na_2S_2O_4$ resulted in the formation of denatured globin protohemochrome only after several hours. From this reason and for the sake of precise reading of the absorption change, the estimation of the reaction product was carried out after converting into its CO-compound after the treatment with NaOH and $Na_2S_2O_4$. The results are shown in Fig. 3. The absorption at 700–800 mμ in the reaction solution of myoglobin was much more intensive than that in hemoglobin reaction. A distinct absorption maximum was observed at 760 mμ, besides which a weak absorption at 710 mμ was also recognized.

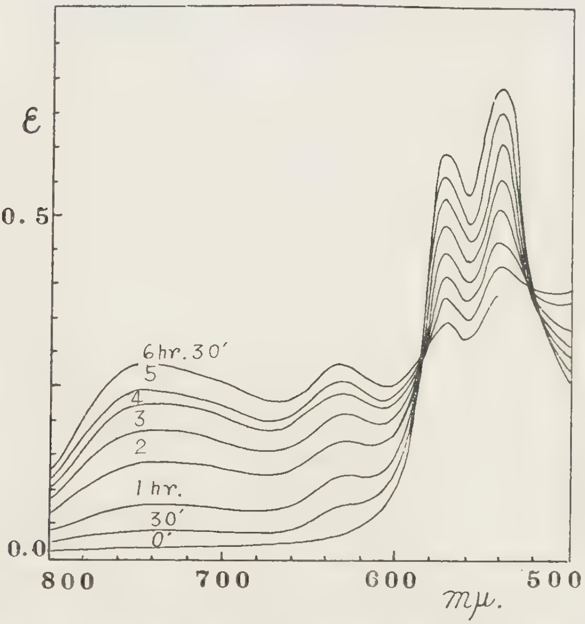


Fig. 3. Absorption change during the reaction process with myoglobin.

2.5 ml.......3×10^{-4} *M* myoglobin. 2.0 ml.......*M*/15 phosphate buffer (pH 7.0). 0.5 ml.......0.1 *M* ascorbic acid (neutralized with NaOH). T : 37°.

Absorption of the reaction solution was measured after the addition of 12 ml. of 1% NaOH with a small amount of powdered $Na_2S_2O_4$ followed by the bubbling with CO gas.

Extraction of Biliverdin—The increase in the yield of biliverdin formed from myoglobin was determined by the method similar to that used in the experiment with hemoglobin. Maximum yield was obtained after 8 hours, namely about 18 per cent of the total heme started. The yield of biliverdin relatively higher than that from hemolobin is in accord with the observation that absorption at 760 mμ was higher in myoglobin experiment.

III. The isolation of Reaction Product by Chromatography

Using active alumina, chromatography was conducted with the reaction solution of myoglobin. Developing with $M/30$ phosphate buffer of pH 7.0, 4 layers of coloured substances were obtained, namely in the following order from the top layer : pink, yellowish green, bluish green, greenish brown as shown by Fig. 4. Each of the coloured substances was extracted from each layer and their absorption figures were determined after the treatment with NaOH and $Na_2S_2O_4$. As shown by Fig. 5. the major part of protoheme was obtained from bottom-most layer including the newly found reaction product showing its characteristic absorption at 700–800 mμ. From the next upper layer, mainly protoheme and choleheme, from the third layer only choleheme were obtained. The pink coloured substance in the uppermost layer was proved to be

FIG. 4

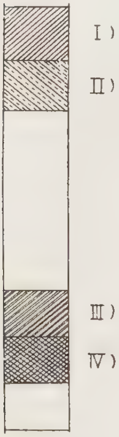


FIG. 5

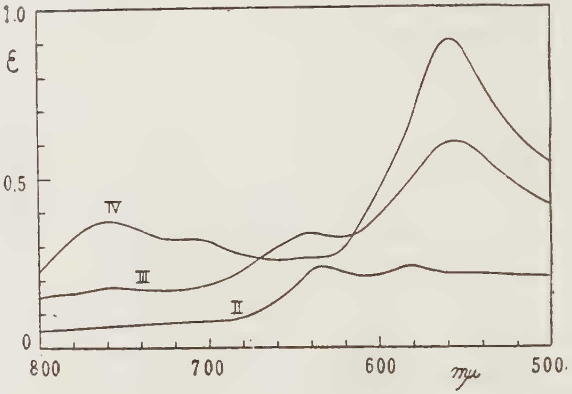


FIG. 4. Chromatography of the reaction products from myoglobin.
I. pink, II. yellowish green, III. bluish green, IV. greenish brown.

FIG. 5. Absorption of the coloured substances extracted from each layer of the chromatography column.

II. from second layer. III. from third layer. IV. from bottom-most layer.

Absorptions of the extracted substances were measured after the treatment with NaOH and $Na_2S_2O_4$.

ascorbic acid. Biliverdin could be extracted only from the bottom-most fraction including minor amount of its oxidized product. No biliverdin could be obtained from other fractions. That the choleheme in the third layer did not move by development, seems to be due to the more progressed denaturation of its protein moiety.

IV. The Identification of the Reaction Products

The Identification of the New Product—The newly isolated compound characterized by its absorption at 760 mμ was identified to be verdoheme. It was assertained, as described above, that the immediate precursor of biliverdin is not the choleheme but is the newly identified substance with its absorption at 700–800 mμ. The new substance is derived from choleheme oxidatively and seems to be structurally similar to verdoheme. In order to clear up the chemical nature of the heme part of the new product, the following experiments were carried out.

The verdohemochrome produced in the reaction system of pyridine hemin—ascorbic acid—O_2 was extracted in chloroform, and washed well with water. From this chloroform solution verdohematin could be extracted, free from pyridine, in 0.1 *N* NaOH (Fig. 6, I). To this solution, globin was added together with NaOH and the solution was reduced by $Na_2S_2O_4$. Then, there appeared an absorption at 700–800 mμ which was precisely the same as that of the newly found product obtained from myoglobin,

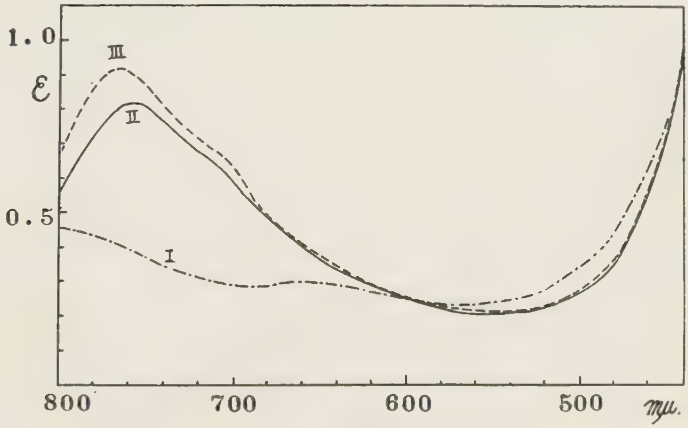


Fig. 6. Absorptions of verdohematin and its derivatives.

I. Verdohematin (ferric) in alkaline solution obtained from verdohemochrome which was prepared by the reaction system of pyridine hemin—ascorbic acid—O_2.

II. Alkali denatured globin verdohemochrome (ferrous).

III. Pyridine verdohemochrome (ferrous) in alkaline solution.

showing its maximum at 760 mμ (Fig. 6, II). When pyridine was added instead of globin, the absorption maximum was at 770 mμ, its extinction being found somewhat higher (Fig. 6, III). From these findings, it seems to be of no doubt that the heme moiety of the final product in the system of myoglobin—ascorbic acid—O_2 is identical with verdoheme.

Chemical Relation of 630-substance to Choleheme—Coleheme in the state of alkali denatured globin cholehemochrme is characterized by its absorption at 618 mμ. On the other hand, 630-substance, the first stage intermediate of pyridine hemin—ascorbic acid—O_2 system, when treated with NaOH and reduced by $Na_2S_2O_4$, shows also its absorption at 618 mμ in the same way as the former (Fig. 7). Thus, it seems to be highly probable that choleheme and the heme moiety of 630-substance are chemically of the same constitution.

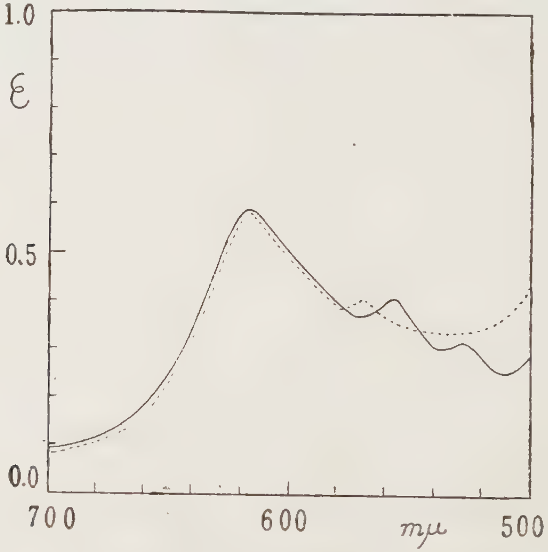


FIG. 7. Comparison of the absorption figures of alkali denatured globin cholehemochrome (full line) and ferrous 630-substance in alkaline solution (dotted line).

From these experimental evidences, it may be concluded that the reaction process of our *in vitro* model system in both globin and hemochrome series proceeds in the same manner in respect to their heme moiety of the starting material in the following two steps :

Protoheme→choleheme→verdoheme.

V. The Quantitative Representation of the Reaction Processes with Hemoglobin and Myoglobin, Respectively

The absorption figures of protoheme and choleheme have been known in their pure state. Now, in respect to the verdoheme, verdohemation obtained from verdohemochrome was transformed into alkali denatured globin verdohemochrome and the absorption figure of the latter was determined in its pure state. Thus, all of the absorption figures of each heme compound in question are known. On the basis of these spectroscopic data, following set of equations was obtained from Fig. 3 showing the absorption change occurring in the reaction process with myoglobin. Applying these equations to the actual absorption figure, each of the heme components existing in the reaction sloution could be calculated.

$$\varepsilon_{573}=x+0.4y+0.25z$$
$$\varepsilon_{630}=0.068x+y+0.37z$$
$$\varepsilon_{760}=0.02x+0.13y+z$$

where x : actual absorption of CO-compound of alkali denatured globin protohemochrome at 573 mμ,

y : actual absorption of CO-compound of alkali denatured globin cholehemochrome at 630 mμ,

z : actual absorption of alkali denatured globin verdohemochrome at 760 mμ.

Thus, each of these heme components, protoheme, choleheme and verdoheme existing at each stage of the reaction process indicated in Fig. 3 was calculated. The results are shown in Fig. 8.

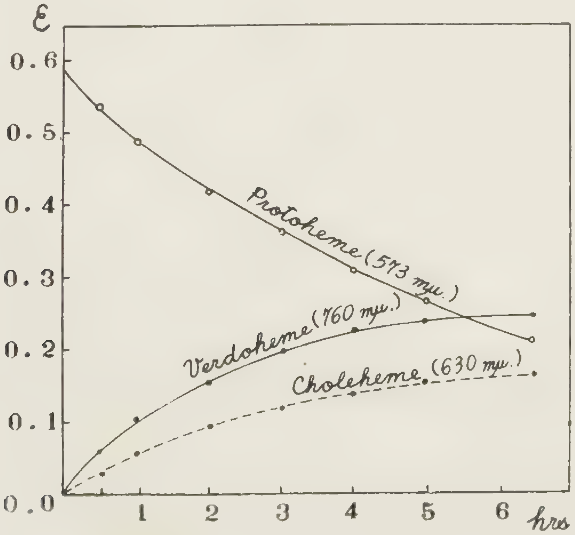


FIG. 8. Quantitative representation of the reaction process of verdohemoglobin formation from myoglobin obtained calculatively from Fig. 3.

By means of an analogous procedure, the following set of equations was obtained from Fig. 1 using hemoglobin.

$$\varepsilon_{557}=x+0.5y+0.25z$$
$$\varepsilon_{618}=0.03x+y+0.37z$$
$$\varepsilon_{760}=0.01x+0.10y+z$$

where x : actual absorption of alkali denatured globin protohemochrome at 557 mμ
y : ,, cholehemochrome at 618 mμ
z : ,, verdohemochrome at 760 mμ

The results of calculation made by applying these equations to Fig. 1, are reproduced in Fig. 9.

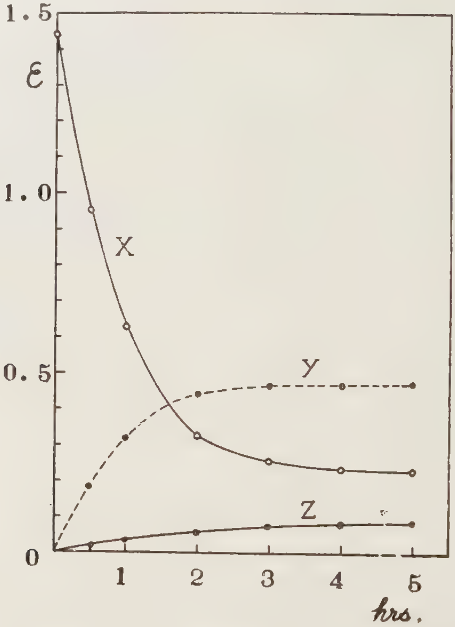


FIG. 9. Quantitative representation of the reaction process of verdohemoglobin formation from hemoglobin obtained calculatively from Fig. 1.

As shown in Figs. 8 and 9, choleheme increased with decrease in protoheme at the initial stage along with the formation of verdoheme. After a certain time of the reaction, however, the production of choleheme remained stationary, while that of verdoheme remained to increase steadily. The formation of verdoheme seemed to have occurred following that of choleheme. Thus, the present reaction may be assumed to be essentially one of the concecutive two step reactions.

The more precise quantitative studies on these reaction process will be reported in the forthcoming communication.

DISCUSSION

Similarity between the Reaction Starting from Pyridine Hemochrome and That from Hemoglobin or Myoglobin—It has been shown unequivocally here that also in the system of hemoglobin (or myoglobin) with ascorbate and oxygen, the reaction proceeds consecutively in two steps, essentially in a similar manner to the analogous system of pyridine hemochrome. In the system of pyridine hemin—ascorbate—O_2, the reaction proceeds in respect to the heme moiety in the following steps: protoheme→630-substance-heme→verdoheme. In the analogous system starting with hemoglobin or myoglobin instead of pyridine hemochrome, the reaction in the heme moiety proceeds according to protoheme→choleheme→verdoheme as has been proved for the first time in the present experiment. In the present system, choleheme has been proved to be the next intermediate to the final product, namely to the immediate biliverdin precursor. Thus, from our examination, it is highly probable that choleheme and 630-substance-heme are of the same chemical structure. The newly found final product in the above mentioned reaction system with hemoglobin (or myoglobin) which was verified by its absorption at 760 mμ will be now denominated as " verdohemoglobin ". The term verdohemoglobin has so far been used rather inconsistently. It should be pointed out here with special emphasis that the immediate precursor of biliverdin is not the choleglobin as has hitherto been generally accepted, but rather its further oxidation product " verdohemoglobin ".

Choleglobin-b—As roughly shown in Figs. 8 and 9, formation of choleheme was found to be higher in the reaction with hemoglobin than with myoglobin. The formation of verdoheme was, however, lower in the former than the latter, accordingly also lower in the yield of biliverdin. This difference seems to be due to the formation of green precipitate (cholehemochrome) during the reaction with hemoglobin. The heme moiety in this green hemochrome consisted purely in choleheme ; it gave no biliverdin by HCl addition, it could not be transferred into ether by the treatment with acetic acid-ether. Choleheme in this compound seems to be strongly bound to protein. The green compound in this state has been denoted as choleglobin-b by Lemberg (*6*). In choleglobin-b, an OH group assumed in α-methene of choleheme has been suggested by Lemberg to be bound with protein-SH. It is well known that myoglobin of horse contains no S atom (*12*), so that if Lemberg's conception were correct and if this were used as the starting material in the present reaction system, choleglobin-b would not be formed. Our experimental result coincided with this presumption; no green precipitate was formed during the reaction in this case and the main portion of the green heme produced in the solution could be transferred into ether when treated with glacial acetic acid and ether. Minor yield of biliverdin in spite of higher production of choleheme in the case of hemoglobin experiment seems to be due to the formation of choleglobin-b which would not be converted into verdohemoglobin.

Reaction Velocity—As seen from the comparison of Figs. 8 and 9, the velocity of heme decomposition in the system with myoglobin was much slower than that with hemoglobin. Oxygen uptake in each of the reactions was measured manometrically by the Warburg technique and compared with each other (Fig. 10). From these data also, the

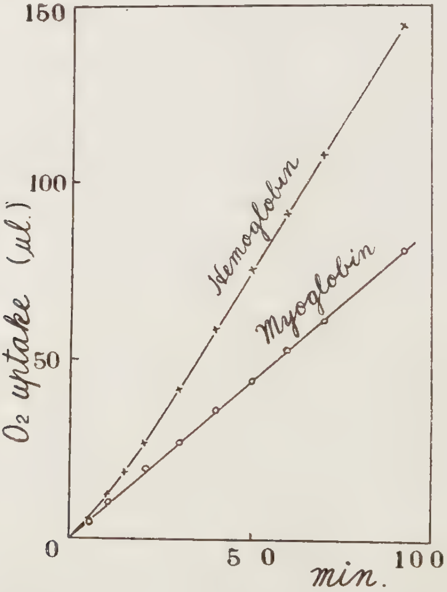


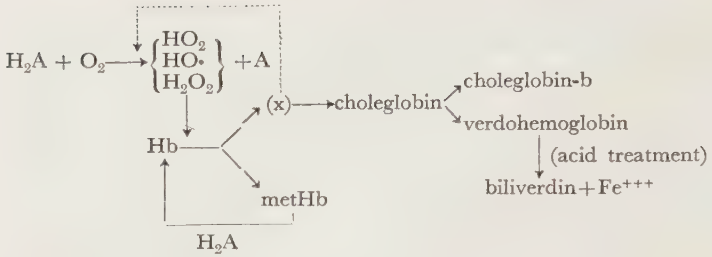
FIG. 10. Oxygen uptake in the reactions with hemoglobin and myoglobin, respectively.

Reaction conditions are the same as those of Figs. 1 and 3, respectively.

reaction rate in the experiment with myoglobin was found to be much lower than that with hemoglobin. It may be pointed out further, as indicated in Fig. 10, that in the initial stage of the reaction with hemoglobin, oxygen uptake increased making a S-curve, though slightly, indicating the existance of an induction phase in the reaction. Such an induction phase of the reaction could, however, not be observable in the case with myoglobin. The difference may be due to the fact, as already known, that the protein moiety of myoglobin is much more resistant against denaturation than that of hemoglobin. As already shown in our previous reports (*13,14*), some denatured hemoglobin molecule is of great significance for the initiation of the reaction in forming choleglobin from hemoglobin. Some explanations should be set forth with respect to this modified hemoglobin molecule. It was demonstrated in our previous papers that the reaction taking place in the formation of choleglobin from hemoglobin is essentially of an autocatalytic chain reaction as represented in the reaction scheme shown in the following section. The reaction seems to be initiated by an oxidase activity of hemoglobin molecule in forming H_2O_2 or related free radicals, so that the rate of the reaction is strongly dependent on the oxidase activity of hemoglobin. An intermediate x assumed in our reaction scheme mentioned in the following section is one of the modified hemoglobin which is much more strongly active as an oxidase than the native hemoglobin. This intermediate is one of the modified hemoglobin molecule, the protein moiety of which is fallen into the state of " perturbation ", a slight and reversible grade of denaturation. The chain reaction nature of the present reaction also seems to be based on the interposition of this intermediate.

The experimental facts mentioned above that the rate of O_2 uptake as well as the rate of heme decomposition were found to be lower in the reaction of myoglobin than in the reaction of hemoglobin can be explained by the stronger resistance of the former against denaturation than the latter, so that the intermediate x will be only diffidently formed in the reaction with myoglobin. The significance of the intermediate x in our representation may thus obtain a further support.

Presentation of the Reaction Scheme—Summing up the experimental facts so far described and those which was preliminary reported, the chemical events in the autocatalytical decomposition of hemoglobin and of myoglobin may be represented by the reaction scheme as follows:



where H_2A: ascorbic acid
A : dehydroascorbic acid

Molecule of hemoglobin, as one of the functional protein, may in some way be affected in its protein moiety during and through its functional activity. Through this distortion in the protein molecule, the functional integrity of the molecule may also be affected according to the grade of substantial alteration. With this functional disintegration of the hemoglobin molecule, the essentially intrinsic oxidase activity of the latter may become prevailing alternatively. This prevailing oxidase activity of the latter may become prevailing alternatively. This prevailing oxidase activity may succeedingly promote the formation of distorted molecule which may lead to the self-decomposition of heme moiety of the activated molecule thus giving verdohemoglobin through choleglobin. Thus, hemoglobin molecule seems to undergo self-decay through its awakened oxidase activity.

SUMMARY

1. In the presence of ascorbic acid, the heme part in the molecules of hemoglobin, myoglobin and pyridine hemochrome underwent aerobic decomposition, all through the same process of two step consecutive reaction, namely: protoheme→choleheme→verdoheme.

2. Verdoheme-protein, when treated with NaOH and $Na_2S_2O_4$, shows a characteristic absorption at about 700–800 mμ (maximum at 760 mμ). Another weak absorption was found at 710 mμ. The new substance was designated by the name " verdohemoglobin ".

3. The immediate precursor of biliverdin which has been formed in this reaction is not choleglobin as has been so far generally accepted, but " verdohemoglobin ".

4. In the experiments with hemoglobin, significant amounts of choleglobin formed during the process were transformed into choleglobin-

b. In this compound, choleheme seems to be bound directly with protein by the SH-group of the latter.

5. Finally, a scheme of probable course of events occurring in the present reaction system was presented on the basis of our experimental findings.

The present work was aided by the grant of the Scientific Research Fund of the Ministry of Education, for which our gratitute is due.

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The Journal of Biochemistry, Vol. 42, No. 4, 1955

STUDIES ON ASPARTASE

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(Received for publication, April 27, 1955)

The deamination of amino acids to acrylic acid was observed in histidine (*1*), tyrosine (*2*), phenylalanine (*3*), and aspartic acid (*4*) respectively. Quastel and Woolf (*4*) demonstrated the reversible reaction, aspartic acid$\rightleftarrows$fumaric acid$+NH_3$ in the presence of *E. coli* and gave the enzyme the name aspartase. Cook and Woolf (*5, 6*) made further observations on the anaerobes. Virtanen and Tarnanen (*7*), and Gale (*8*) purified cell free aspartase from B. fluorescence and *E. coli* respectively by means of autolysis, isoelectric precipitation and salting out method.

Ichihara *et al.* (*9*) obtained inactive purified histidine deaminase, which converts histidine to urocanic acid, and demonstrated the restoration of activity by the addition of folic acid and SH-glutathione. Based on this fact, the following experiments were carried out on the aspartase of *E. coli.*

EXPERIMENTAL

Materials

Strain—E. coli communior was supplied by the Microbiological Institute of Osaka University. This strain was cultured on agar slants (pH 7.4) consisting of 10 g. each of beef extract and peptone, 2 g. NaCl, 30 g. agar and 1 liter of distilled water. Each of the strains thus obtained from isolated cultures was inoculated on a medium of the following composition : 10 g. each of dried yeast and peptone, 5 g. K_2HPO_4 and 1 lit. of water. After boiling the medium for one hour water was added to make the original volume, the pH of the filtrate was adjusted to 7.2 and the solution was heated again at 120° for 15 minutes. The strain was grown in shake cultures at 37° for 12–14

This work is supported by a Grand for Scientific Research by the Department of Education and this paper was presented at the Meeting of Biochemistry, Kinki Division, Oct., 1953.

hours. After adding 0.85 per cent NaCl solution, the suspension was centrifuged repeatedly in order to wash the cells, the paste spreaded in a film on a glass plate and dried in a vacuum dessicator at −10°.

Enzyme Preparations—The dried cells were ground with equal amounts of quartz sand and suspended in $N/10$ phosphate buffer (pH 8.0) (40 mg./1 ml.) and kept at 4° for one hour. After centrifuging (3000 r.p.m.) for 30 minutes the supernatant was used as a crude enzyme solution.

The crude extract was subjected to fractionation with saturated ammonium sulfate. Each fraction was dissolved in minimal volume of water and dialyzed by use of a collodion membrane for 72 hours until the Nessler's test was negative. The 50–60 per cent fractions, as shown later, retained the highest activity of ammonia production. This fraction was dissolved in minimal volume of chilled water and was treated with 4 per cent calcium phosphate-gel for one hour at pH 6.0, 4°. The adsorbant was separated from the supernatant at 3000 r.p.m. and washed with chilled water until no traces of NH_3 remained. The supernatant removed from the adsorbant was dialyzed and was shown to have no activity. The adsorbant was eluted in 1 M phosphate buffer (pH 7.5), equal to the volume of the fraction, for 18 hours. The eluate was separated from calcium phosphate-gel by centrifuging at 3000 r.p.m. for 10 minutes and used as a purified enzyme.

L-*Aspartic Acid*—A preparation of the Takeda Pharmaceutical Ind. Ltd.

$CoCl_2$—A preparation of the Merck Pharmaceutical Co. Ltd.

Methods

Enzyme Reaction and Determination of NH_3—This was carried out using a reaction flask of 30–40 ml. capacity with side arm. The technique was as follows :

Main compartment : Enzyme solution, 1.0 ml. or 20 mg. of dried cells ; 1 M phosphate buffer (pH 7.2), 1 ml. ; L-aspartic acid (7.5 μM/ml.), 1 ml. ; distilled water, 0.7 ml.

Side arm : 1 N H_2SO_4, 1.0 ml.

The above reaction mixture was incubated at 37° in N_2-atomosphere (O_2 free by alkaline pyrogallol solution) for 2 hours (18 hours for the cell suspension). To the control flask was added 1 ml. of water in place of the substrate. At the end of the reaction, the contents of both chambers were mixed. To the test flask was added 1.0 ml. of distilled water and to the control 1.0 ml. of aspartic acid solution and the mixtures were centrifuged (3000 r.p.m., 30 minutes) to remove protein precipitant. Aliquot of the supernatant was used for determination of NH_3.

According to the method of Archibald (*10*), using Nessler's reagent, NH_3 was photometrically determined with filter S_{47}. As the standard solution, 2 ml. of 0.0005 M $(NH_4)_2SO_4$ ($N=28.016\ \gamma$) was used. In the following tables, the amount of ammonia formed is indicated in percentage to the theoretical value of substrate.

Paper Chromatography—The developing solvent was a mixture of *n*-butanol, glacial acetic acid and distilled water (4:1:1). Spots developing on the filter paper were tested with a spray of 0.04 per cent of brom phenol blue in 100 ml. of ethanol.

Determination of Oxaloacetic Acid—This was carried out by the anilin-citrate method of Coopenhauer (*11*).

RESULTS

Activity of the Preparations—As shown in Table I, the ammonium formation activity of the living cells is higher at anaerobe than at aerobe. Subsequent experiments were, therefore, carried out anaerobically.

Optimal pH—Since the ammonium formation was maximum at pH 7.0 to 7.5, subsequent experiments were carried out at pH 7.3.

TABLE I

Ammonia Formation by Living Cells, Dried Cells and Cell Extracts.
Ammonia formation was indicated in percentage to the theoretical value of the substrate.

	NH_3 formed (%)		
	Living cells, incubated for 18 hours.	Dried cells, incubated for 2 hours.	Cell extract, incubated for 2 hours.
Aerobe	57.5		
Anaerobe	80.0	77.1	87.0

Fractionation with $(NH_4)_2SO_4$—As shown in Table II, the maximum activity of ammonium sulfate fractions was found to lie in the 50 to 70 per cent fractions.

TABLE II

Activity of Fractions Fractionated with Ammonium Sulfate

NH_3 formed (%)			
Cell extract	Fractionated with saturated ammonium sulfate		
	0-50 %	50-70 %	70-100 %
50	0.6	20	2

Restoration of Activity of Enzymes by Supplements—As shown in Table III, the eluate, which had been partially or completely inactive, regained its activity by the addition of both $CoCl_2$ and folic acid most intensively

(52–82 per cent). Though 60 per cent restoration of activity was seen with $CoCl_2$ alone in the case of the preparations retaining yet a week activity, 8 per cent recovery was seen in the case of the inactive eluate.

The restoration was also seen by addition of $CoCl_2$ with SH-glutathione, but it was much less (53 per cent) than by addition of $CoCl_2$ with folic acid (82 per cent), and moreover the addition of SH-glutathione decreased the effect of the latter (74 per cent).

TABLE III

Restoration of the Activity of Enzymes by Addition of $CoCl_2$, Glutathione and Folic Acid

Addition	NH_3 formed			(%)
	I	II	III*	Remarks
None	12	0	0	1 mg. folic acid was dissolved in 1 ml. of $M/10$ phosphate buffer (pH 7.5). 0.2 ml. of this solution was used. Final concentration, 6.9×10^{-9} M. 5 mg. of SH-Glutathione was dissolved in 1.0 ml. of phosphate buffer and 0.2 ml. of this solutions was used. Final concentration, $9 \times 10^{-9} M$.
Folic acid	14	/	5	
SH-glutathione	8	/	/	
$CoCl_2$	61	/	8	
Folic acid+SH-glutathione	/	6	/	
$CoCl_2$+folic acid	/	82	52	
$CoCl_2$+SH-glutathione	/	53	/	
$CoCl_2$+folic acid+SH-glutathione	65	74	/	

* The eluate was dialyzed at 4° 48 hours, using a collodion membrane, against 500 times of water.

Saturation of Folic Acid and $CoCl_2$ for the Formation of Ammonia—Table IV and V show that for the formation of ammonia, folic acid and Co-ion have saturation effect to each other in order to restore the maximum activity of the enzyme.

Paper Chromatography of the Reaction Product—For this purpose, twice the amount of enzyme solution described above was used. $CoCl_2$ and folic acid were added. The initial activity of the preparation was 11 per cent and this increased to 51 per cent when supplemented with $CoCl_2$ and folic acid.

After the reaction, one fourth of the solution was used for determination of ammonia and the rest (3/4) was used for chromatography. The deproteinized supernatant, separated by centrifuging, was dried

TABLE IV

Saturation of Folic Acid for $CoCl_2$

Addition		NH_3 formed (%)
$CoCl_2$ final concn.	Folic acid	
None	None	3
2×10^{-5} *M*	None	25
”	20 γ	49
”	50	47
”	100	48
”	200	42
”	400	42
”	800	47

TABLE V

Saturation of Co-ion for Folic Acid

Addition		NH_3 formed (%)
Co ions.	Folic acid	
None	None	1.3
None	20 γ	3.6
20 γ	”	33
50	”	40
100	”	42
300	”	42
500	”	42

in a vacuum dessicator and exracted with 1 ml. of 98 per cent alcohol. The alcohol extract was used for paper chromatography as described above. As the control test, 2.0 ml. of fumaric acid (7.5 ml.) was added to the reaction flask prior to the reaction instead of aspartic acid and treated similarly. The spot on the filter paper of the reaction product was in accord with that of fumaric acid (Rf. 0.87).

Investigation of Oxaloacetic Acid—Fumaric acid may possibly be formed in the present reaction through oxaloacetic acid. The activity of the enzyme preparation used here was 26 per cent (ammonia formation) and this was increased to 90 per cent by the addition of $CoCl_2$ and folic acid. After the reaction, one-half of the solution was used for the determination of ammonia and the other half for the determination of oxaloacetic acid. Oxaloacetic acid was determined by the modified aniline-citrate method of Coopenhauer using the Warburg's manometer. Carbon dioxide could not be proved. Accordingly, the formation of fumaric acid from oxaloacetic acid was excluded.

Purity of the Enzyme Preparations—The eluate was dialyzed by use of collodion membrane against 500 times water at 4° for 48 hours and aliquots were used for determination of nitrogen by the microKjeldal method of Parnas (*12*). The remaining part of the solution was used for the reaction as described above after addition of $CoCl_2$ and folic acid (53 per cent ammonia formed). Tne crude enzyme solution (cell extracts) which formed 53 per cent ammonia, was also used for determination of nitrogen. It was 27.6 times purified (Table VI).

TABLE VI

Purity of Purified Enzyme

		Crude extract	Purified eluate
Volume	*ml.*	0.5	1.0
NH_3 formed	%	53	53
N content	γ	1293.8	46.9
Purity		27.6 times	

Behabior of Inhibitors—The activity of the crude enzyme was completely inhibited by $HgCl_2$ and $AgNO_3$; 44 per cent by CH_2ICOOH, 42 per cent by α,α'-dipyridyl, 20 per cent by each of NaN_3 and KCN, 10 per cent by oxin; NH_2OH, $H_2N\text{-}CONHNH_2$, Na_3AsO_3 and NaF had no inhibitory effect (Table VII).

Behavior of Bivalent Metal Ions—Of the bivalent metal ions tested the Co-ion was most active and the others were much less active (Table VIII).

DISCUSSION

From the above data, it was confirmed that folic acid was also essential for the activity of aspartase of *E. coli*, as was found in histidine deaminase. However, in aspartase the Co-ion was required in addition

Talbe VII

Behavior of Inhibitors on the Enzyme Activity

0.3 ml. of 10^{-2} M solution of each inhibitor was added and the total volume was made up to 3.0 ml. Final concentration was 10^{-3} M.

Inhibitor	Inhibition*
	%
NH_2OH	0
$H_2NCONHNH_2$	0
NaN_3	20
Oxin	10
KCN	20
α,α'-Dipyridyl	42
$HgCl_2$	100
$AgNO_3$	100
CH_2ICOOH	44
Na_3AsO_3	0
NaF	0

* Parallel experiments with and without addition of inhibitor were made at the same time.

The difference between ammonia formation of both experiments was indicated in percentage to that of the controll.

Table VIII

Behavior of Bivalent Metal Ions

Addition (final concn. 10^{-3} M)	NH_3 formed (%)	
	Not added	Added
$FeSO_4$	30	43
$CoCl_2$	16	64
$NiSO_4$	68	74
$ZnSO_4$	68	83
$MgCl_2$	16	22
$CuSO_4$	55	70
$MnSO_4$	55	74

to folic acid. Recently, Koizumi *et al.* (*13*) repeated the experiments on histidine deaminase in which ethylendiamine tetraacetate was added and confirmed that in histidine deaminase, folic acid, SH-glutathione and Co-ion were also essential for its activity.

SH-Glutathione markedly enhanced the effect of folic acid on histidine deaminase, but as shown in Table III, SH-glutathione showed an inhibitory tendency on the effect of Co-ion and folic acid in the case of aspartase. According to our other experiment, however, the inactive aspartase obtained by dialyzing cell extract for 72 hours, regained its activity by 100 per cent by the addition of these all three. Moreover the enzyme preparation, which retained activity, regained its activity by addition of Co-ion and SH-glutathione (Table III). Consequently the effect of SH-glutathione on aspartase can not be desregarded.

Among the bivalent metal ions investigated, only the effect of Co-ion was marked. The enzyme preparation which was obtained by Sakamoto (*14*), however, regained its activity by the addition of Cd-ion as well as by Co-ion. Suda *et al.* (*15*) reported, that highly purified histidine deaminase regained its activity by the addition of Cd, Hg or Zn ion. As Table VIII shows, other metal ions have some activity. It therefore can not be concluded that the Co-ion is unique as the activator.

SUMMARY

1. Aspartase has been obtained from *E. coli* in a soluble and purified state. From nitrogen determinations it was found to have been purified 27.6 times.

2. The optimum pH for the enzyme activity is 7.0–7.5 ; the activity of 50 to 70 per cent fraction, fractionated with ammonium sulfate, is the highest.

3. The activity is inhibited completely by $HgCl_2$ and $AgNO_3$; partially by α, α'-dipyridyl, momoiodoactic acid and slightly by KCN and NaN_3 but not by oxin, NH_2OH, $H_2NCONHNH_2$, Na_3AsO_3, and NaF.

4. The purified enzyme regains its activity when supplemented with both Co-ion and folic acid and a definite concentration of each effective for the maximum restoration of the activity was demonstrated. Other bivalent metal ions are much less effective. The effects of SH-glutathione and Cd-ion were discussed.

5. Fumaric acid was confirmed as the reaction product by means of paper chromatography.

6. Oxaloacetic acid was not proved as a reaction product.

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